Proceedings
of the
Society
for
Experimental Biology and Medicine

Vol. 85 April, 1954 No. 4

SECTION MEETINGS

CLEVELAND, O.	
University Hospitals	February 8, 1954
DISTRICT OF COLUMBIA	
George Washington University	April 1, 1954
ILLINOIS	
Chicago Medical School	February 16, 1954
MINNESOTA	
University of Minnesota	March 24, 1954
SOUTHWESTERN	
University of Texas Medical School	March 5-6, 1954

Monolayer Tissue Cultures. II. Poliomyelitis Virus Assay in Roller Tube Cultures of Trypsin-Dispersed Monkey Kidney.* (20940)

J. S. Youngner. (Introduced by Jonas E. Salk.)
From the Virus Research Laboratory, University of Pittsburgh School of Medicine.

The purpose of this report is to present data on the utilization of trypsin-dispersed monkey kidney cells prepared according to a modification(1) of the method of Dulbecco and Vogt(2) for titration of poliomyelitis virus in roller tube cultures. Data are presented on yield of satisfactory culture tubes and on the experience with titrations of poliomyelitis viruses in such cultures as compared with cultures containing tissue fragments.

Methods and materials. Preparation and standardization of trypsin-dispersed cell suspension, as well as materials employed, are the same as described earlier(1), including Medium D used to obtain outgrowth. At the time of virus inoculation, *Medium E,* composed of a mixture of 97.5 ml of Synthetic mixture 199 and 2.5 ml of 5% $NaHCO_3$/100 ml, was employed to replace medium D. Penicillin and streptomycin were added to a concentration/ml of 100 units and 0.1 mg, respectively. Medium D may be used to advantage when longer survival of cultures is desired, but was omitted in these studies to avoid any possible influence of the horse serum component on virus activity. *Preparation of roller tube cultures of kidney fragments.* Kidney fragments about 1-2 mm in diameter were prepared by mincing with scissors and were planted without exposure to trypsin. Two types of kidney fragment cultures were utilized: (a) kidney fragments grown under perforated cellophane(3), and (b) kidney fragments grown directly on the glass wall of the roller tube. From 5 to 10 fragments of kidney cortex were introduced into each tube and outgrowth obtained by

---

* Aided by a grant from The National Foundation for Infantile Paralysis, Inc.

TABLE I.
Culture Tube Production from 18 Successive Batches of Trypsin-Dispersed Cell Suspension.

Date	Cell susp. stored 4°C (days)	Dil. of cell susp.	Approx. No. cells/tube (in 0.5 ml)	No. tubes prep.	No. tubes discard	% usable
			×1000			
7/17/53	0	1:100	314	290	0	100
9/ 3	0	1:200	350	199	9	95
4	0	1:200	500	40	2	95
		1:400	250	91	14	84
8	0	1:100	600	174	1	99
11	0	1:300	310	64	1	98
16	0	1:260	300	100	2	98
22	0	1:200	270	101	4	96
25	0	1:300	400	300	0	100
29	3	1:200	380	46	2	95
11/ 9	1	"	600	300	5	98
12	1	"	300	306	15	95
16*	1	"	470	312	26	91
20*	1	"	500	216	29	87
23*	1	"	550	300	30	90
12/ 3*	1	"	550	595	78	86
8	1	"	620	600	86	85
14*	1	"	310	300	34	88
15*	1	"	570	231	16	93

* Kidneys from rhesus monkeys employed in the preparation of batches marked with asterisk. All other batches prepared with tissue from cynomolgus monkeys.

incubation while rolling at 36°C; nutrient consisted of 2 ml of Medium D/tube. After 6 or 7 days a zone of healthy epithelial outgrowth surrounded one or more fragments in each tube. At that time, the medium used to obtain outgrowth of epithelial cells was removed and replaced by Medium E, and inoculations carried out as described below. *Inoculation of culture tubes with virus.* Using either 0.1 ml or 0.5 ml as inoculum, prepared using Medium E as diluent, the final volume of medium was brought to 2 ml in each tube with Medium E. Following inoculation, they were placed in a roller drum, since it has been found that with rolling, virus-induced cellular degeneration appeared more rapidly and non-specific degenerative changes occurred less frequently than when inoculated cultures remained stationary. Microscopic examination for signs of virus-induced destruction was carried out at 3 and 6 days following inoculation. Tubes showing typical signs of virus-induced degeneration were considered infected regardless of the degree of severity of the cellular changes. Titers were calculated according to the method of Reed and Muench(4) and were expressed as the number of 50% infectious doses (ID_{50})/ml of original virus pool, based on the dilution of virus added to the cultures. *Viruses employed.* The strains of virus used in these studies were Mahoney (Type 1), MEF-1 (Type 2), and Saukett (Type 3); the histories of these strains and their adaptation to tissue culture have been described previously(5).

Results. Yields of satisfactory cultures from successive batches of trypsin-dispersed cell suspension. Table I contains a summary of the results obtained with culture tubes prepared from 18 batches of trypsin-dispersed cell suspension. It can be seen that 85-100% of cultures were usable when freshly prepared suspension or suspension stored for one day at 4°C was employed; in one instance, suspension stored for 3 days was utilized. With certain batches of cell suspension, viability has been maintained after storage for as long as 7 days, the longest period studied. Suspensions have been shipped by air to cities 300 to 900 miles away, and have been transported by train, both without refrigeration; after arrival, suspensions were found to be usable for providing satisfactory cultures. The majority of cultures discarded were unsatisfactory because of incomplete sealing of

TABLE II. Summary of Data Comparing Infectivity of Poliomyelitis Viruses in Different Types of Kidney Cultures.

Cultures employed	Neg. log ID_{50} doses per ml		
	Type 1 Mahoney (pool #50)	Type 2 MEF-1 (pool #60)	Type 3 Saukett (pool #70)
Roller tubes			
Trypsin-dispersed cells	6.9	6.6	6.7
Fragments on glass	6.2	5.9	5.8
Fragments under cellophane	5.5	5.4	5.6
Petri dishes			
Trypsin-dispersed cells	7.7*	7.1	7.1

* Negative log of the number of plaque-forming particles/ml.

the tubes due either to defective caps or to a cracked lip on the screw-capped tube. Such incomplete seals permitted the pH to rise and remain above 8.0, thus preventing growth.

In tubes with complete closure, the pH of the medium dropped to 6.8 on the 6th or 7th day of incubation when 0.5 ml volume of medium was employed. With experience it was found that this pH drop could be used as a reliable indication of a usable tube without the necessity for microscopic examination. It was usually sufficient to discard all alkaline tubes and spot check microscopically only every 10th tube showing an acid pH. With amounts of medium greater than 0.5 ml/tube, the pH drop could not be used to aid in selection of usable cultures. For work requiring more precise observations all tubes were examined microscopically prior to inoculation.

Assay of poliomyelitis viruses in roller tube cultures of trypsin-dispersed monkey kidney cells. Comparison with titers obtained using kidney fragment cultures. Two to four replicate titrations of pools of virus strains representing each of the 3 immunologic types of poliomyelitis virus were carried out in 6- or 7-day-old roller tube cultures of trypsin-dispersed monkey kidney cells as well as in cultures of monkey kidney fragments; 4 to 10 cultures were used for each virus dilution. In each instance, higher virus titer was noted in roller tube cultures of trypsin-dispersed kidney cells than in the cultures employing fragments. The data summarized in Table II indicate that from 1.0 to 1.5 logs more virus activity was demonstrable when dispersed-cell cultures were used than when cultures of kidney fragments under cellophane were employed. The sensitivity of roller tube cultures of kidney fragments on glass was from 0.7-0.9 log less than that of cultures prepared with trypsin-dispersed cell suspension.

Using a technic similar to that recommended by Dulbecco and Vogt(2), plaque forming particle (p.f.p.) counts were done with the 3 virus pools employed in these studies. As a matter of interest, the counts obtained are included in Table II. In all cases, the number of plaque-forming particles/ml of original virus pool was somewhat greater than the number of ID_{50} doses/ml. The details of the method employed and a more detailed comparison of ID_{50} titers in roller tubes and p.f.p. counts in petri dishes will be made in a subsequent report.

Discussion. In attempting to understand the differences in sensitivity to virus encountered in the different kinds of cultures, the following factors may apply. In kidney fragment cultures, a proportion of tissue fragments do not grow out and with inocula containing only a few infectious particles, it is conceivable that these tissue fragments may adsorb and remove enough infectious particles so that multiplication of virus may not occur. With trypsin-dispersed cell cultures, the epithelial monolayers contain no large tissue fragments and all but a few of the cells are viable and susceptible, presenting a much reduced opportunity for adsorption of a virus to inert tissue material; it is likely that such cultures provide a greater opportunity for contact between virus and a susceptible cell, a factor of importance when few infectious virus particles are concerned.

Summary. The preparation of roller tube cultures of trypsin-dispersed monkey kidney cells and their utilization for the assay of poliomyelitis viruses has been described. Data have been presented that show that cultures prepared in the manner described are more sensitive indicators of the presence of poliomyelitis virus infectivity than are cultures prepared from minced kidney fragments.

1. Youngner, J. S., PROC. SOC. EXP. BIOL. AND MED., 1954, v85, 202.

2. Dulbecco, R., and Vogt, M., *J. Exp. Med.*, 1954, v99, 167.

3. Youngner, J. S., PROC. SOC. EXP. BIOL. AND MED., 1953, v84, 697.

4. Reed, L. J., and Muench, H., *Am. J. Hyg.*, 1938, v27, 493.

5. Youngner, J. S., Ward, E. N., and Salk, J. E., *ibid.*, 1952, v55, 301.

Received February 15, 1954. P.S.E.B.M., 1954, v85.

Role of Reticulo-Endothelial System and Disposition of Dietary Cholesterol in Various Mammalian Species.* (20941)

LAWRENCE FEIGENBAUM, SANFORD O. BYERS, AND MEYER FRIEDMAN.
From the Harold Brunn Institute, Mount Zion Hospital, San Francisco, Calif.

In a recent report from this laboratory(1), it was shown that the hepatic R-E cell is essential in the disposition of *exogenously* derived cholesterol in the rat. This was demonstrated not only by microscopic detection of cholesterol in the Kupffer cell within 6 hours following administration of cholesterol, but also by the production of hypercholesteremia and persistence of a chylomicronemia in rats given parenteral injection of various colloidal suspensions believed capable of interfering with hepatic reticulo-endothelial cell function.

In view of these results, it was decided to determine whether this hypercholesteremia following interference with the integrity of the R-E system could be duplicated in other species than the rat. Therefore, the present study was undertaken in which rabbits and dogs, as well as rats, received India Ink intravenously together with high cholesterol feedings.

Effect of intravenous administration of India Ink on plasma cholesterol and total lipid of animals on high cholesterol feedings. Plasma samples of all species studied were taken before and 48 hours after the beginning of the experiment. These were analyzed for their total cholesterol and total lipid content by methods previously described(2,3). In addition, most of the samples were examined microscopically under darkfield illumination for the presence of chylomicra.

Rats. Methods. Male rats of the Long-Evans strain, approximately 15 weeks old (avg wt 244 g), were used. Two series of rats were studied. Series A (15 rats) was given 100 mg of cholesterol in 3 cc of olive oil by stomach tube per day for 2 days. Series B (13 rats) was given 100 mg of cholesterol in 3 cc of olive oil by stomach tube intubation per day, *plus* 0.5 cc of 20% India Ink intravenously twice daily for 2 days.

Results. The control rats given cholesterol feedings alone, showed a rise in plasma cholesterol (Table I) but the average plasma cholesterol concentration at 48 hours was still only 64 mg/100 cc and the plasma lipid concentration was not found to change. However, when India Ink was injected into the rats of Series B in addition to the oral administration of cholesterol, a striking increase in both their plasma cholesterol and total lipid occurred. Thus, the average plasma cholesterol rose from 52 mg % before the experiment to 100 mg/100 cc 48 hours after the beginning of the experiment. The average plasma lipid content rose from 242 to 322 mg/100 cc in the same period of time.

As observed earlier(1), chylomicra invariably were absent from the sera of the control rats and always present in great numbers in the sera obtained at 48 hours from ink-injected rats. The latter sera, also, were invariably turbid.

---

* This work was aided by grants from the Life Insurance Medical Research Fund and the National Heart Institute, National Institutes of Health, Public Health Service.

TABLE I. Effect of Injection of India Ink upon the Plasma Cholesterol and Lipid Content of Rat, Rabbit and Dog after Ingestion of Cholesterol in Olive Oil.

			Plasma cholesterol (mg/100 cc)		Plasma lipid (mg/100 cc)	
	Series	No. of animals	Before feeding	48 hr after feeding	Before feeding	48 hr after feeding
			Rats			
Avg	A	15*	49	64	170	173
Range			(33–74)	(40–82)	(133–200)	(148–250)
S.E. mean			± 2.5	± 2.9	± 10.0	± 11.2
Avg	B	13†	52	100	242	322
Range			(32–74)	(79–140)	(157–295)	(227–388)
S.E. mean			± 3.3	± 4.8	± 13.4	± 12.8
			Rabbits			
Avg	A	8*	53	204	107	354
Range			(32–90)	(129–251)	(73–135)	(218–545)
S.E. mean			± 5.6	± 4.5	± 6.4	± 12.4
Avg	B	10†	73	316	137	1323
Range			(46–152)	(158–558)	(91–204)	(435–4100)
S.E. mean			± 9.0	± 33.6	± 10.4	± 14.6
			Dogs			
Avg	A	7‡	161	217	360	455
Range			(102–196)	(167–262)	(208–477)	(317–568)
S.E. mean			± 11.4	± 11.2	± 36.3	± 28.4
Avg	B	8‡	164	258	460	738
Range			(115–196)	(210–304)	(310–640)	(640–820)
S.E. mean			± 8.6	± 12.9	± 34.9	± 19.0

* Animals given 400 mg cholesterol/kg/day dissolved in olive oil.
† *idem,* but oil and India Ink.
‡ Dogs given 600 mg cholesterol/kg/day dissolved in olive oil.

Rabbits. Methods. Male rabbits, approximately 16 weeks old, were used. Two series of rabbits were studied. Series A (8 rabbits) was given 400 mg of cholesterol (33.5 mg cholesterol/cc of olive oil) per kg body weight by stomach tube daily for 48 hours. Series B (10 rabbits) received the same amount of cholesterol, *plus* 5 cc of 20% India Ink suspension intravenously daily for 48 hours.

Results. As was anticipated, the increment of increase in the plasma cholesterol of both Series A and Series B (Table I) was greater than that in the rat experiments. However, once again there was a greater increase (333%) of plasma cholesterol in those rabbits receiving the R-E "blocking agent," India Ink, than that (285%) observed in the rabbits which received only the cholesterol in olive oil feedings. The total plasma lipid content also showed a more marked increase in those animals receiving the intravenous India Ink.

Unlike the control rats, the control rabbits fed only cholesterol, exhibited many chylomicra in their plasma at the 48-hour period. However, even more chylomicra were observed in the plasma of the rabbits fed cholesterol and injected with India Ink.

Dogs. Methods. Two series of mature mongrel dogs were used. Series A (7 dogs), the control series, received cholesterol (0.6 g in 10 cc olive oil/kg body weight) by gastric intubation daily for 2 days. Series B (8 dogs) received an intravenous injection of one cc of 20% India Ink solution/kg body weight per day, in addition to the above-described gastric feedings of cholesterol in olive oil. The India Ink was given within 2 hours following the administration of the cholesterol feeding. Blood samples, taken before and 48 hours after the beginning of the experiment, were analyzed and examined as described above.

Results. The results of these experiments indicated that the dog exhibited a moderate increase in both its plasma cholesterol and total lipid content following the administration of cholesterol in oil alone. Thus (Table I), the average plasma cholesterol and lipid content of the 7 control dogs was 161 and 360 mg/100 cc respectively before, and 217 and 455 mg/100 cc 48 hours after feeding of cholesterol alone. This represents an increase of 35 and 26% respectively in the cholesterol and lipid plasma content. The experimental series of 8 dogs, however, given the same amount of cholesterol in oil, plus intravenous injection of India Ink, exhibited an even greater rise in both the cholesterol and total lipid content of their plasma. Thus the average plasma cholesterol increased (Table I) from 160 to 258 mg/100 cc (an increase of 57%) and the average plasma total lipid increased from 460 to 738 mg/100 cc (an increase of 60%).

As in the rat, the plasma samples obtained at the 48-hour period from the ink-injected dogs, showed turbidity as well as myriads of chylomicra, whereas the plasma samples of the control dogs were clear and showed few or no chylomicra.

Discussion. The present observations indicate that the intravenous injection of India Ink, when *combined* with the gastric intubation of cholesterol dissolved in olive oil, leads to frank hypercholesteremia in the rat, intensifies the expected hypercholesteremia of the rabbit and elevates the plasma cholesterol of the dog. The plasma lipid of these 3 species is similarly affected by the combination of ink injection and cholesterol feeding. These results suggest that our earlier finding of the essentiality of the hepatic reticulo-endothelial system for proper metabolism of *dietary* cholesterol in the rat(1) is a phenomenon not limited to this species, because when this system is interfered with in other mammalian species, the latter show an interference in their usual handling of cholesterol.

Earlier studies of this laboratory demonstrated that the liver functions to remove and store the excess cholesterol in the plasma derived from the diet(4) until it is later either discharged again into the plasma or converted and excreted into the bile as bile salt(5,6). However, almost all dietary cholesterol after absorption from the intestine travels in intestinal lymph enmeshed in chylomicra(7) and the relatively huge size of these particles makes it almost impossible to conceive of them as being able to make their way through the intact capillary walls of the liver to the hepatic parenchymal cells. Obviously then, they must either be broken down in plasma (*e.g.* solubilized by "clearing factor") or ingested by a phagocytic cell and solubilized intracellularly. It therefore was no surprise to us to find(1) that the hepatic Kupffer cell and only this type of reticulo-endothelial cell of the rat contained cholesterol demonstrable by Sudan staining and refraction of polarized light soon after the oral ingestion of this steroid. Apparently then, this cell ingests the chylomicron and in so doing solubilizes the cholesterol component contained therein and then transfers it to the hepatic cell. It was the discovery of this cell's similar handling of the lipid content of the chylomicron which caused Gilbert and Jomier(8) to call the Kupffer cell, "l'auxiliare précieux et dévoué de cellule hépatique"—a function later confirmed by Jaffé and Levinson(9). The injection of a "blocking agent" simultaneously with the absorption of dietary cholesterol interferes with this ingestion process and in so doing leads to a hypercholesteremia ostensibly due to the retention in plasma of chylomicron cholesterol. This interference process also explains the frequent associated finding of plasma turbidity and may be responsible for the results recently obtained by LeQuire, Gray, and Cobb(10) who observed that following injection of thorotrast in the dog a marked reduction in the clearing of lipemic plasma occurred following heparin injection.

Summary. Interference with the reticulo-endothelial system by injection of India Ink was found to interfere with the ability of the rat, rabbit and dog to clear their plasma after the ingestion of cholesterol dissolved in olive oil. This interference extended not only to cholesterol, but also to total lipid and chylomicra. These findings suggest that the reticulo-endothelial system plays an essential role in the disposition of exogenously derived cholesterol. The possible mechanism involved is discussed.

---

1. Friedman, M., Byers, S. O., and Roseman, R. H., *Am. J. Physiol.*, April, 1954.

2. Byers, S. O., Friedman, M., and Michaelis, F.,

J. Biol. Chem., 1950, v184, 71.

3. Friedman, M., Byers, S. O., and Michaelis, F., *Am. J. Physiol.*, 1951, v164, 786.

4. Friedman, M., Byers, S. O., and Shibata, E., *J. Exp. Med.*, 1953, v98, 107.

5. Byers, S. O., and Biggs, M. W., *Arch. Biochem.*, 1952, v39, 301.

6. Friedman, M., Byers, S. O., and Gunning, B., *Am. J. Physiol.*, 1953, v172, 309.

7. Byers, S. O., Friedman, M., and Gunning, B., In preparation.

8. Gilbert, A., and Jomier, J., *Arch. de med. exp.*, 1908, v20, 156.

9. Jaffé, R. H., and Levinson, S. A., *Am. Rev. Tbc.*, 1928, v11, 217.

10. LeQuire, V. S., Gray, M. E., and Cobb, C. A., *Circ. Res.*, 1953, v1, 523.

Received February 19, 1954. P.S.E.B.M., 1954, v85.

Inhibition of Hyaluronidase Activity by Factors Associated with Blood Clotting. (20942)

KARL L. ROTH. (Introduced by D. R. Meranze.)

From the Department of Research, Albert Einstein Medical Center, Southern Division, Philadelphia, Pa.

The interaction of hyaluronidase with the clotting mechanism has been suspected for some time. Attempts have also been made to identify complement (C') with the nonspecific serum hyaluronidase inhibitor and also with prothrombin(1-5). This suggests a possible relationship between the prothrombin complex and inhibitor. Baserga *et al.*(6) noted that hyaluronidase interferes with the prothrombin consumption in sera. The inhibition of the agglutination of platelets by this enzyme was observed by Quattrin(7). Fiala *et al.*(8,9) have shown that dialyzed bovine hyaluronidase acts as an anticoagulant *in vitro*, by virtue of its capacity to counteract the thromboplastic effect of platelets. This interaction between platelets and hyaluronidase was postulated as representing a competition between the two, for a specific plasma protein(9). The latter was found to be stable on storage, and to have the same adsorption affinities as prothrombin, but was nevertheless present in a prothrombin free serum. Fiala has further stated(10) that tissue thromboplastin inhibits the enzymatic degradation of hyaluronic acid. Studies made in this laboratory on the interaction between hyaluronidase and the clotting mechanism have revealed that this phenomenon is more complex than can be explained merely as a competitive inhibition between platelets and enzyme. The data to be presented below suggest that the nonspecific serum (plasma) inhibitor of hyaluronidase may play a specific role in the mechanism of blood coagulation.

Materials and methods. Platelets were isolated by differential centrifugation from plasma containing Sequesterine^R (ethylene-diamine-tetraacetic acid), and collected in siliconized vessels. The final centrifugate was washed 4-fold with saline, resuspended in distilled water and then shaken for 5 minutes with aid of a mechanical shaker. The shattered and agglutinated platelets were separated by centrifugation at low speed, and the supernatant "platelet extract" poured off. The sediment was washed once by shaking with one-third volume distilled water and the wash water combined with the supernate. The final sediment was discarded. This platelet extract was found to correct the clotting

TABLE I. The Effect of Proconvertin Concentration on "Anticoagulant" Activity of Hyaluronidase.

Proconvertin eluate added (ml)	0	.1	.2	.3	.4	.5	Control
Clotting time on recalcification (sec.)	∞	1800	1450	1100	1000	1040	1060

Each tube contained 0.5 ml plasma with 3×10^4 platelets, 0.1 ml hyaluronidase and saline plus proconvertin eluate, total 1.1 ml. In the control saline replaced enzyme. 0.5 ml (0.025M) $CaCl_2$ added after 10 min. incubation at 37°C. Clotting times are the avg of 3-4 determinations.

TABLE II.

Plasma added:	0	Dic. 1	Dic. 2	Dic. 3	Normal	Dic. 1 Dic. 2 only	Control (no enz.)
Clotting time on recalcification (sec.)	∞	3300	2900	2500	2100	∞	1700
Prothr. times of added plasmas (sec.)		28.2	21.4	17.7	12.4		12.4

Each tube contained .2 ml platelet poor test plasma, .1 ml enzyme and .2 ml correcting plasma or saline to make a volume of .5 ml. Calcium, and additional saline, where necessary, added after 10 min. incubation at 37°C. Final volume: 0.9 cc.

defect of a platelet-free plasma. The activity of the preparation was resistant to boiling for 5 minutes and to treatment with 10% trichloracetic acid, and subsequent neutralization with NaOH. Whenever intact platelets were required, the washed saline suspension, kept in siliconized test tubes, was used. Any desired platelet number was achieved by simple dilution of the suspension with additional saline.

Proconvertin. In some experiments aged serum was used as a source of proconvertin (11). The addition of thromboplastin, as suggested by Owren, was omitted. For most work, however, a concentrate prepared by adsorption of aged serum with $BaSO_4$ or $Ca_3(PO_4)_2$ and elution with sodium citrate (0.02 M) was used. This is referred to as proconvertin eluate. In the studies of the effect of proconvertin on the depolymerizing activity of hyaluronidase, the eluate was dialyzed not against saline, as in the clotting studies, but against tap water. Human brain thromboplastin, prepared for routine use in this laboratory, served as thromboplastic reagent. Commercial preparations of hyaluronidase from Armour & Co. (Chicago, Ill.), G. D. Searle & Co. (Chicago, Ill.), Ortho Pharmaceutical Co. (Raritan, N. J.), and Wyeth Inc. (Philadelphia, Pa.) were used. Hyaluronic acid was obtained from Armour & Co.* and General Biochemical, Inc. (Chagrin Falls, O.). The enzyme solution was employed after dialysis against distilled water or saline. These materials were assayed viscosimetrically with an Ostwald type viscosimeter (vol. 5 cc) at 37°C. Oxalated plasmas were used in clotting studies. Sodium citrate was found to inhibit to some extent the anticoagulant effect of hyaluronidase.

Results. A platelet poor plasma, on incubation with hyaluronidase in a concentration of one mg/ml of plasma, becomes incoagulable. This defect can be corrected by addition of either tissue thromboplastin or platelets (9). Furthermore, it will be shown that addition of proconvertin will also correct the clotting defect. A normal plasma also loses its ability to clot on recalcification if a sufficiently high concentration of hyaluronidase is added to it. It has already been demonstrated that this "anticoagulant" effect of the enzyme appears to be inversely proportional to the number of platelets in the plasma(8). However, our experiments show (Table I) that if the platelet concentration is kept constant, and the level of proconvertin varied, the "anticoagulant" effect of hyaluronidase appears to be related to the concentration of proconvertin. The proconvertin effect is also illustrated in Table II, in which the behavior of 3 dicoumarolized plasmas (proconvertin poor plasmas) is compared with that of a normal plasma, relative to their capacity toward correcting the hyaluronidase clotting defect. The data given in Table II show that the decreased level of proconvertin in dicoumarolized plasmas is able to rectify only in part the clotting inhibition by hyaluronidase, the degree of rectification being inversely related to the prothrombin time of the plasmas.† If hyaluronidase is initially incubated with platelets(9), tissue thromboplastin or proconvertin eluate, its anticoagulant activity is lost; simultaneously the clotting factors also lose part or all of their activity.

---

* We are indebted to Dr. L. L. Lachat for generous supplies of hyaluronic acid.

† Preliminary experiments show that the level of the nonspecific serum hyaluronidase inhibitor is depressed in patients receiving dicoumarol. The inhibitor level tested in sera of two hemophiliacs was found to be normal.

TABLE III. Reciprocal Inactivation of Clotting Factors and Hyaluronidase.

Factor present:	0	Hyaluronidase	Platelet extract	Proconv. eluate	T'plastin (1:100)
A. Recalcified clotting times/sec. *before* incubating factors with hyaluronidase	800	2100	95	610	35
B. Recalcified clotting times *after* incubating factors with enzyme	—	—	890	880	760
C. After boiling 1 min.	—	—	98	—	70*

A.—0.1 ml of given factor or enzyme added to 0.1 ml of platelet poor test plasma and the volume made up to 0.3 ml with saline. After bringing temperature to 37°C, 0.1 ml of 0.025M $CaCl_2$ was added and the clotting time determined.

B.—Equal volumes of enzyme and factors were mixed, then 0.2 ml added to 0.1 ml plasma. After a short incubation at 37°C recalcified clotting times were determined as in A.

C.—(*) The activity of a 1:200 saline dilution of above thromboplastin preparation was 68, 72 and 69 sec. after 1 min. boiling.

This reaction between the enzyme and clotting factors occurs very rapidly, without an irreversible change of platelets or thromboplastin; for when the mixture of either one of these 2 factors is boiled for one minute, the activity of the factors is recovered. (Table III). With proconvertin, however, the reaction with enzyme gives rise to an irreversible alteration of the proconvertin, as reflected by a loss of specific proconvertin activity, the alteration proceeding at a slow, but definite rate, and marked by the recovery of the anticoagulant effect of the enzyme. To demonstrate this, hyaluronidase was incubated with a small excess of proconvertin eluate. Initially the mixture did not exhibit any anticoagulant effect when added to a test plasma, but after the third hour of incubation the mixture caused a marked prolongation of the clotting time, showing the gradual recovery of the hyaluronidase effect (Fig. 1). On the other hand, loss of proconvertin potency was demonstrated by the fact that whereas a 1:10 dilution of tissue thromboplastin in saline easily overcomes the anticoagulant effect of hyaluronidase (shown by the normal prothrombin times), after several hours of incubation of enzyme with plasma, a gradual prolongation of prothrombin times was observed. Addition of as little as 0.05 cc of a proconvertin eluate brought the prothrombin times back to normal. Control tubes showed that only a small fraction of the prothrombin time prolongations was to be attributed to a possible decrease in ACG level (Fig. 2). The capacity of proconvertin preparations and of tissue thromboplastin to inhibit hyaluronidase activity was also demonstrated by viscosimetric studies. 0.1 mg of hyaluronidase‡ in 0.1 ml H_2O and 0.1 ml of clotting factor were added to 3.0 ml of hyaluronic acid in the viscosimeter at 37°C. After suitable intervals the flow time of the mixture was determined to give a measure of the hyaluronidase activity present. As can be seen in Fig. 3, tissue thromboplastin and proconvertin inhibit the depolymerizing action of the enzyme. Boiled thromboplastin possessed some inhibitory activity also. Curiously, a mixture of platelets and proconvertin was a more effective inhibitor than proconvertin alone, even though platelets alone were inactive. It was also

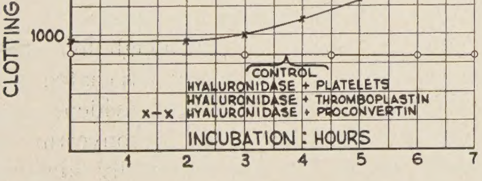


FIG. 1. Effect of prolonged incubation of hyaluronidase with factors on clotting time of a normal plasma. Mixtures were those described in Table III (B). At noted time intervals aliquots (0.2 cc) were added to 0.1 ml test plasma and clotting time determined after a short incubation.

---

‡ Approx. 20 V.U.

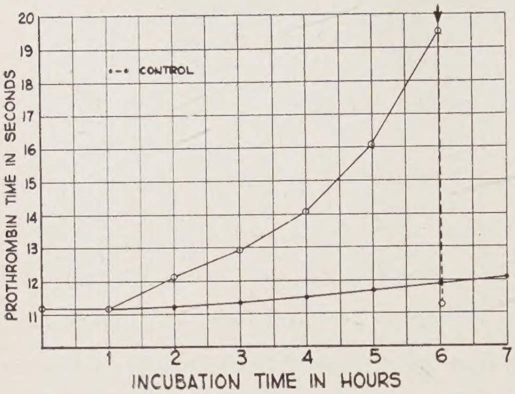


FIG. 2. Alteration of prothrombin time of a test plasma on prolonged incubation with hyaluronidase. 2 ml plasma were incubated with 0.4 ml hyaluronidase. Aliquots were taken at intervals indicated and prothrombin times determined with a 1:10 saline dilution of tissue thromboplastin-Ca. At time shown by arrow, 0.2 cc proconvertin eluate was added to the remaining plasma.

observed that sera freed of all proconvertin activity by adsorption on $Ca_3(PO_4)_2$, $BaSO_4$, $Mg(OH)_2$ or inactivated at 56°C for 30 minutes, possess no inhibitory activity.

Discussion. It is generally agreed that nonspecific serum (plasma) hyaluronidase inhibitor is a factor (or factors) present in the normal circulatory system. Glick *et al.*(12) find the inhibiting property to be "inherent in various products of liver metabolism" . . . It is established that hyaluronidase inhibitor is stable on storage, that it has adsorption affinities similar to prothrombin but is, however, present also in a prothrombin free serum. Its blood level seems to be decreased by dicoumarol administration. Furthermore, all active proconvertin preparations are able to inhibit hyaluronidase. These observations are consistent with the conclusion that the inhibitor and proconvertin may be one and the same substance.

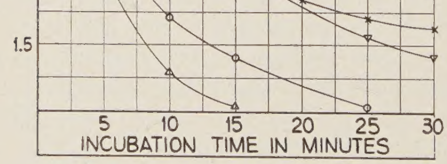


FIG. 3. Effect of various factors on rate of depolymerization of hyaluronic acid.

On the other hand, in the course of a routine experiment it was observed that storage of a proconvertin preparation for several days at —20°C, resulted in the loss of approximately 50% of its initial hyaluronidase inhibiting activity, without any parallel loss in its blood clotting (proconvertin) activity. A similar phenomenon was observed by Fulton *et al.*(4) in their investigation of properties common to hyaluronidase inhibitor and Complement (C′). Intact complement complex was required for hyaluronidase inhibition. However, on lyophilization, guinea pig serum was found to lose its inhibitory activity without suffering any loss in its complement titer. With both complement and proconvertin preparations, the hyaluronidase inhibiting activity lost on freezing can be promptly restored by the addition of inactivated serum, which possesses neither complement nor proconvertin, and very little, if any, inhibitory activity. This suggests that full inhibition depends on the presence of a heat stable second factor, a co-factor, which is present in inactivated serum, but is destroyed by freezing. Such a cofactor has already been postulated by Fulton *et al.*

Furthermore, the experimental results presented in Fig. 3 show that a proconvertin eluate, previously mixed with platelet material, has a slightly higher inhibitory activity than the proconvertin preparation untreated. This activity is stable on storage at room temperature. Platelets alone are inactive.

Thus, it appears that although hyaluronidase inhibition is intimately associated with proconvertin activity, present evidence suggests that inhibitor and proconvertin are closely related but not chemically identical. The inhibitor may be a complex; composed of proconvertin, a heat stable co-factor and another factor, perhaps of platelet origin.

It has been shown that both platelets and tissue thromboplastin are able to counteract the anticoagulant effect of hyaluronidase, even after boiling for 5 minutes. However, only thromboplastin is able to inhibit the degradation of hyaluronic acid by hyaluronidase, boiled preparations being slightly less active than unboiled. Inactivation by incubation with "Thromboplastinase"(13) of a thromboplastin preparation, to the extent of reducing its clotting potency from 12″ to 160″, also did not affect its inhibitory activity. Even a crude alcohol-ether extract of acetone dried brain tissue was active.

Thus, the level of inhibiting material present in tissue thromboplastin is not affected, and remains essentially unchanged, despite chemical alteration of the thromboplastin to the degree that its plasma coagulation capacity is greatly decreased.

Present understanding of the phenomena involved do not permit a decision as to whether this hyaluronidase inhibiting component present in tissue thromboplastin complex is similar to the hyaluronidase inhibitor in blood. But the observations reported in this paper do show that an intimate relationship exists between some of the components of the blood coagulation reaction chain and the enzyme, hyaluronidase.

Summary. 1. Proconvertin preparations were found to function as hyaluronidase inhibitors. The inhibition of hyaluronic acid depolymerization by proconvertin requires a cofactor and is enhanced by prior incubation with platelet material, which, when tested alone, is inactive. 2. Tissue thromboplastin is also shown to inhibit hyaluronidase. This inhibitory activity seems to be inherent mainly in the lipid moiety, and although always associated with the fraction containing the thromboplastic principle, is independent of its activity. 3. It is suggested that the nonspecific serum hyaluronidase inhibitor is associated with a part of the thromboplastic complex of the blood.

Appreciation is expressed to Dr. D. R. Meranze, Director of Research, for his kind encouragement and interest.

This work was supported by grants No. RG 2927C from the National Institute of Health and No. S-23 from the Albert Einstein Medical Center, Philadelphia.

1. Wilander, O., *Scand. Arch. f. Physiol.*, 1939, Suppl. XV.

2. Felix, Pendel, Roka, *Hoppe Seyler Z. f. Physiol.*, 1949, v284, 198.

3. Mann, Hurn, PROC. SOC. EXP. BIOL. AND MED., 1948, v67, 83.

4. Fulton, Marcus, Robinson, *N. Y. Acad. Sci.*, 1950, v52, 7.

5. Marcus, Fulton, *Fed. Proc.*, 1948, v8, 407.

6. Baserga, de Nicola, Vahi, *Experientia*, 1950, v6, 12.

7. Quattrin, *Il Farmaco*, Napoli, 1950.

8. Fiala, Meranze, Roth, *Science*, 1952, v115, 2996.

9. Fiala, Roth, *Fed. Proc.*, 1952, v11, part 1 (*ibid.* oral communication)

10. ———, *Arch. Int. Physiol.*, 1953, v61, 205.

11. Owren, *Scand. Journ. Clin. and Lab. Inv.*, 1951, v3, 3.

12. Snively, Glick, *J. Clinical Inv.*, 1950, v29, 1087.

13. Gollub, Feldman, Schecter, Kaplan, Meranze, PROC. SOC. EXP. BIOL. AND MED., 1953, v83, 853.

Received February 26, 1954. P.S.E.B.M., 1954, v85.

Revival of Tubercle Bacilli After Prolonged *in vitro* Exposure to Isoniazid. (20943)

Myron W. Fisher. (Introduced by A. C. Bratton, Jr.)
From the Research Laboratories, Parke, Davis & Co., Detroit, Mich.

Although there have been several publications(1-3) in which evidence was presented that isoniazid is apparently bactericidal for tubercle bacilli *in vitro,* experiments performed in these laboratories and elsewhere(4) have indicated that under certain conditions isoniazid is not bactericidal. Since it has been demonstrated(5) that tubercle bacilli in a resting as well as a growing state will rapidly and strongly absorb and bind isoniazid, this uptake and associated cellular damage may not be easily reversible under the usual test conditions. In such an event, isoniazid-treated mycobacteria would be called "dead". However, it is the purpose of this communication to describe experiments which have demonstrated that when a suitable isoniazid antagonist is employed, revival of tubercle bacilli can be accomplished even after exposure for 5 weeks to amounts of isoniazid 100,000 times greater than the minimal growth inhibitory concentration. These revived cells were not isoniazid-resistant, nor, as far as could be determined, did they represent a small fraction of the original population.

Methods. All experiments were performed in liquid media and with submerged suspensions of tubercle bacilli. Resting cell suspensions of the H37Rv strain of *Mycobacterium tuberculosis* var. *hominis* were obtained by taking a 14-day growth in Dubos liquid Tween-albumin medium which was washed and suspended in phosphate buffer containing 1000 γ of isoniazid/ml. This buffer consisted of 0.63% $Na_2HPO_4 \cdot 12H_2O$ and 0.1% KH_2PO_4, adjusted to pH 6.8. Replicate volumes of the isoniazid-buffer mixture were inoculated with H37Rv so that about 2 x 10^8 cells/ml were present. These were held at room temperature for 5 weeks, and at frequent intervals nephelometric readings were made to determine whether an appreciable amount of growth or lysis had occurred(6). At weekly intervals, an aliquot of isoniazid-treated cells was washed twice in the buffer and then inoculated in a liquid basal synthetic medium* (BM)(7), BM plus 10% (v/v) bovine serum, and BM plus various concentrations of some compounds which were known to antagonize growth inhibition by isoniazid of the H37Rv strain. These compounds were hemin, potassium ferricyanide, sodium pyruvate, and manganous chloride(7). The various types of media inoculated with isoniazid-treated cells were incubated at 37°C for a maximum of 35 days, with inspections made at 7-day intervals for the detection of growth. Any growth which occurred in revival media after 5 weeks exposure to 1000 γ isoniazid was subcultured in Dubos liquid Tween-albumin medium and tested for isoniazid sensitivity in the basal medium and a serum-enriched medium.

Results. Turbidimetric measurements indicated that there was neither growth nor lysis of cells during the 5-week isoniazid treatment. As shown in Table I, reversal of isoniazid inhibition throughout the 5-week period was accomplished in media containing hemin, ferricyanide, or pyruvate. The least concentrations which effected this reversal were: hemin, 0.63 γ/ml; ferricyanide, 25.0 γ/ml; and pyruvate, 500.0 γ/ml. No reversal was apparent in the basal medium or in media containing $MnCl_2$ in concentrations ranging from 10.0 through 0.02 γ/ml. After 2 weeks exposure to INH, growth did not occur in the serum medium. Although precise quantitative methods were not applied, there appeared to be no significant effect on the growth rate or total growth of control H37Rv cultures by the addition to the basal medium of the aforementioned concentrations of hemin, ferricyanide, or pyruvate. On the other hand, as indicated in Table I, the growth of "revived" cells was progressively slower and scantier following subculture from isoniazid exposure.

---

* In per cent: asparagine, 0.1; $Na_2HPO_4 \cdot 12H_2O$, 0.63; KH_2PO_4, 0.1; sodium citrate, 0.15; $MgSO_4 \cdot 7H_2O$, 0.15; glycerol, 2.0. pH 6.8.

TABLE I. Growth of *M. tuberculosis* after INH Exposure.

	Growth* in					
Wks exposed to INH	Basal medium (BM)	BM + serum	BM + hemin	BM + $K_3Fe(CN)_6$	BM + pyruvate	BM + $MnCl_2$
1	0	4+	4+	3+	4+	0
2	0	4+	4+	3+	4+	0
3	0	0	3+	2+	+	0
4	0	0	3+	2+	+	0
5	0	0	3+†	+†	+†	0

* Approximated by visual inspection.
† Tested for INH sensitivity.

This was particularly the case with media containing ferricyanide or pyruvate.

As indicated in Table I, growth which occurred after 5 weeks of isoniazid treatment was tested for isoniazid sensitivity. The minimal inhibitory concentration of isoniazid for all such cultures was from 0.006 to 0.024 γ/ml, which is identical with that obtained for the "parent" H37Rv strain(7).

Discussion. A salient feature of this study, in addition to the failure to detect a pronounced bactericidal action of isoniazid, is the illustration of one fallibility of any experiment for the detection of a bactericidal action. It was demonstrated long ago by Chick(8), in the case of heavy metals and sulfides, that a pseudo-bactericidal effect may be observed if one does not employ an antagonist. In this instance, had the revival tests been conducted with only the basal medium or in the serum-enriched medium, it would have been concluded that isoniazid had "killed" all cells within 2 weeks.

It may be argued that isoniazid, like penicillin, is bactericidal only for growing cells, and that these experiments merely confirm this hypothesis. However, it should be pointed out that: (a) resting cells will firmly bind isoniazid(5) and it may be assumed that this bound isoniazid will contribute largely if not entirely to subsequent growth inhibition; (b) by the criteria previously employed, *i.e.*, without an antagonist in the revival medium, the "resting" isoniazid-treated cells in this study would be classed as "dead"; and (c) additional experiments, not fully described here, were performed with growing H37Rv cells with essentially similar results. The latter experiments were performed only with hemin as a reversing agent, which was added directly to H37Rv cultures held at 37°C for 3 weeks in basal media and containing from 0.02 through 10.0 γ isoniazid/ml. The final hemin concentration was 100 γ/ml, as used here, and the highest isoniazid concentration reversed was 2.5 γ/ml. It should also be mentioned that if as little as 0.01% "Tween 80" was added to the menstruum in which tubercle bacilli were exposed to isoniazid, no reversal (revival) was accomplished by any of the antagonists previously named for either resting or growing cells.

The nature or mechanism of isoniazid reversal is not yet clear. The failure of Mn^{++} to reverse isoniazid inhibition may be a matter of cell impermeability. It is likely, as indicated by chemical studies, that the effect of ferricyanide may be ascribed to a direct destruction of isoniazid through oxidation. On the other hand, since hemin and pyruvate are important metabolites, their reversal of isoniazid activity may have been achieved through some reactivation or by-pass of the metabolic site which was damaged or blocked by isoniazid. Because it has been shown by the author(9) that certain isoniazid-resistant strains require hemin as a growth factor, and, more recently, that pyruvate or α-ketoglutarate may also have this function, the hypothesis is strengthened that the site of action of isoniazid may be directly related to the porphyrin metabolism of tubercle bacilli(7), and indirectly to their carbohydrate metabolism.

Summary. Tubercle bacilli exposed to 1000 γ isoniazid/ml of a buffer medium for 5 weeks at room temperature may be revived by appropriate concentrations of hemin, potassium ferricyanide, or sodium pyruvate. It was concluded that isoniazid possesses only a bacteriostatic effect under these conditions.

The technical assistance of Misses Noraldeen Palecek and Katherine Byrnes is gratefully acknowledged.

---

1. Steenken, W., Jr., and Wolinsky, E., *Am. Rev. Tuberc.*, 1952, v65, 365.

2. Middlebrook, G., *Am. Rev. Tuberc.*, 1952, v65, 765.

3. Mackaness, G. B., and Smith, N., *Am. Rev. Tuberc.*, 1953, v67, 322.

4. Grundland, I., *Compt. rend. acad. sci.*, 1953, v236, 152.

5. Barclay, W. R., Ebert, R. H., and Koch-Weser, D., *J. Lab. Clin. Med.*, 1953, v42, 779.

6. Fisher, M. W., Kirchheimer, W. F., and Hess, A. R., *J. Bact.*, 1951, v62, 319.

7. Fisher, M. W., *Am. Rev. Tuberc.*, 1954, v69, 469.

8. Chick, H., *J. Hyg.*, 1908, v8, 92.

9. Fisher, M. W., Montross, F. L., and Palecek, N. M., *Am. Rev. Tuberc.*, 1952, v66, 626.

Received February 26, 1954. P.S.E.B.M., 1954, v85.

Exogenous Androgen and Non-Directed Hyperexcitability in Castrated Male Guinea Pig.* (20944)

JEROME A. GRUNT. (Introduced by J. W. Everett.)

From the Departments of Anatomy, University of Kansas, Lawrence, Kansas, and Duke University School of Medicine, Durham, N. C.†

In the course of an investigation of the effects of castration and subsequent therapy with testosterone propionate‡ on the sexual behavior of the male guinea pig(1,2), a type of excited non-sexual behavior became increasingly evident. This behavior, called *non-directed hyperexcitability*, could be best described as a sudden straightening of the limbs, jumping into the air, and frequently, by turning of the head. These actions occurred suddenly and did not fit into any sequence of behavior usually seen. The excited movements seemed to occur without reference to the female or to the other behavior seen during the test.

Since this phenomenon is seen periodically in the intact guinea pig, little attention was given to it at the start of the experiment. However, as the investigation progressed it became evident that an increase in the frequency of this behavior had taken place following castration. As a result, systematic observations were not begun until after the main experiment was well underway.

Method. The procedures used in this experiment have been described(1,2). Briefly each of a group of young, mature male guinea pigs 80 to 150 days of age was isolated in an individual cage and given weekly tests in order to determine the strength of sex drive(3). Following the tenth test, all but the intact controls were castrated. After 16 weeks, during which time sexual activity fell off to a low level, all of the castrates except those used as controls were divided into evenly matched groups and given varying amounts of testosterone propionate for the remaining 15 weeks of the experiment. As indicated above, a portion of the experiment had already been completed before the present work was begun. Twenty-eight male guinea pigs of the larger group used in the original experiments contributed data to the present study; data were collected after the third week following castration. These 28 males were divided in the following manner: 2 were intact controls; 7 were given 12.5 γ testosterone propionate/

---

* This investigation was supported by research grants from the National Institutes of Health, U. S. Public Health Service; the Committee for Research in Problems of Sex, National Research Council, administered by Dr. William C. Young of the Department of Anatomy, University of Kansas School of Medicine; and the University of Kansas Research Fund.

† Present address.

‡ Testosterone propionate was supplied by the courtesy of Ciba Pharmaceutical Products, Inc.

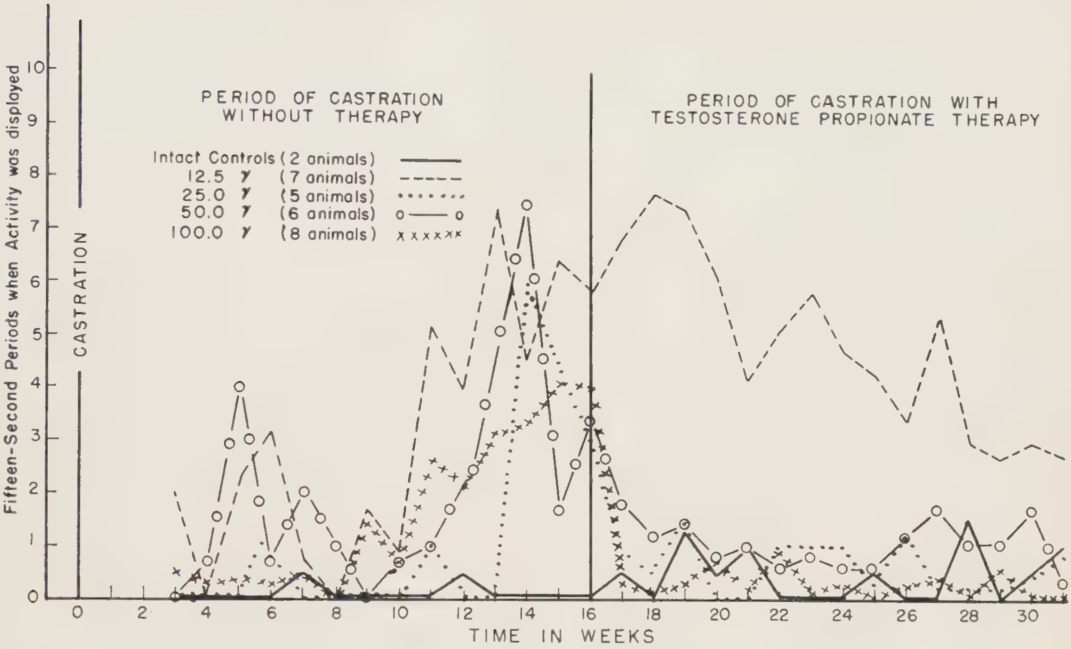


FIG. 1. Non-directed hyperexcitability of male guinea pig after castration and following androgen therapy.

100 g body weight daily starting the 17th week following castration; 5 were given 25 γ; 6 were given 50 γ; and 8 were given 100 γ of androgen. For purposes of scoring the sexual behavior, each minute of the test was divided into quarters. In the present study this approach was utilized; the number of 15-second periods in which incidents of non-directed hyperexcitability occurred were noted and were plotted in relation to time.

Results. Only infrequently during the 28 weeks in which observations were made did the intact controls display this behavior in more than one 15-second period per test (Fig. 1). In contrast, all 4 groups of castrates displayed increasing amounts of this behavior, showing it in at least four 15-second periods during tests prior to the beginning of androgen therapy.

Following the injection of testosterone propionate, 17 weeks after castration, the frequency of occurrence of the non-directed hyperexcitability decreased sharply in the groups receiving 25 γ of the androgen or more. Within a week these animals displayed this behavior at approximately the same level as the intact controls. The group given 12.5 γ testosterone propionate/100 g body weight daily differed from the others. Administration of this amount of androgen decreased the frequency of non-directed hyperexcitability to a lesser degree. Even at the end of the experiment this group displayed the behavior in an average of 2.7 15-second periods of the last test.

Discussion. What appear to be comparable data have been presented for the rat(4). Beach, using castrated male rats, injected with either estrogen or progesterone, showed that there was an inverse relationship between the amount of extra-copulatory excitement, and the vigor and frequency of masculine copulatory responses. The masculine copulatory behavior in turn was related to the levels of injected estrogen.

It appears that the behavior here described is also inversely related to the level of administered steroid; and that the administration of an amount of testosterone propionate greater than 12.5 γ/100 g body weight/day is required to prevent its abnormal manifestation.

Interestingly enough, these data coincide with those obtained for the sexual behavior

(1,2). It was shown that when 25 γ or more testosterone propionate was injected, the sexual behavior was comparable with that of the intact controls; while the group receiving 12.5 γ always had a lower drive. As in the part of the study summarized earlier it was not possible to differentiate between those animals receiving daily injections of 25, 50, or 100 γ testosterone propionate/100 g body weight.

Summary. A non-directed hyperexcitability, dependent on the level of androgen, has been described in the male guinea pig. An increase in the frequency of its occurrence took place after castration; and with the daily administration of testosterone propionate in amounts of 25 γ or more per 100 g body weight there was an immediate return to the level of the intact control. Injection of 12.5 γ of the hormone did not eliminate the increase which followed castration. The similarity of these results to other data bearing on the effect of testosterone on sexual behavior is noted.

---

1. Grunt, J. A., and Young, W. C., *Endocrinology,* 1952, v51, 237.

2.———, *J. Comp. Physiol. Psychol.,* 1953, v46, 138.

3. Young, W. C., and Grunt, J. A., *ibid.,* 1951, v44, 492.

4. Beach, F. A., *Endocrinology,* 1942, v31, 679.

Received March 4, 1954. P.S.E.B.M., 1954, v85.

Effect of Quinones on Adaptive Enzyme Formation in a Strain of *Pseudomonas aeruginosa.* (20945)

FREDERICK BERNHEIM.

From the Department of Physiology and Pharmacology, Duke University Medical School, Durham, N. C.

A washed suspension of a strain of *Pseudomonas aeruginosa* oxidizes benzoic acid after a latent period of 100-120 minutes(1). The latent period can be decreased and the rate of benzoate oxidation increased by the previous addition of succinate and ammonium sulfate. These effects are proportional, within limits, to the amount of succinate added(2). As a result of the oxidation of succinate, ammonia is assimilated, precursors, possibly peptides or proteins(3) are stored in the cell, and these can subsequently be utilized to form the enzyme when benzoic acid is added. It was thus possible to study the effect of a compound on enzyme formation in 2 ways provided the rate and extent of succinate oxidation is not affected. If the compound is added at the beginning with succinate and ammonia, it could increase or decrease the formation of the precursors and, if it is not metabolized, also affect the utilization of the precursors for enzyme formation. If the compound is added with the benzoate after the succinate oxidation is completed, it could only affect the utilization of precursors. The following is a study of the effect of catechol, protocatechuic acid and related compounds on the formation of the enzyme for benzoic acid.

Methods. A strain of *Pseudomonas aeruginosa* originally obtained from Dr. Koppers was grown in 100 ml of nutrient broth (Difco) for 48 hours at 37°C. The cells were centrifuged down, suspended in 50 ml distilled water, centrifuged again, and finally suspended in 7.0 ml of 0.05 M Na-K-phosphate buffer pH 7.8. 0.5 ml of this suspension was used in each Warburg vessel which had a final fluid volume of 2.0 ml. All the compounds added were dissolved in buffer and when necessary adjusted to pH 7.8. 0.2 mg of succinic acid and 0.2 mg ammonium sulfate were added to the bacteria at zero time with or without the compound to be tested and the rate and extent of the oxygen uptake measured. The succinate oxidation was usually completed in one hour. At the end of 2 hours, 1.0 mg of sodium benzoate was added from the side arm with or without the

TABLE I. Effect of Various Compounds on Oxidation of 1 mg of Sodium Benzoate. Cells were incubated 2 hr at 37°, pH 7.8 with 0.2 mg succinic acid and 0.2 mg $(NH_4)_2SO_4$. Compounds were added (a) at zero time with succinic acid and ammonia, or (b) at the end of 2 hr with the benzoate. In the concentrations used none of the compounds affected the rate or extent of succinate oxidation. Figures, in mm³ oxygen uptake, represent oxidation of benzoate from which the appropriate control uptakes have been subtracted.

γ/ml	Compound added	mm³ O_2 uptake				
		60 min.	75 min.	90 min.	105 min.	120 min.
	0	11	49	113	201	304
50	Catechol with benzoate	6	8	9	13	14
25	” ” ”	10	27	44	72	113
5	” ” succinate	26	78	116	282	401
10	” ” ”	57	132	238	359	481
15	” ” ”	66	142	246	365	487
50	Protocatechuate with benzoate	29	87	147	244	350
5	” ” succinate	22	81	150	253	371
10	Dopa with benzoate	1	5	15	48	96
10	” ” succinate	13	52	118	210	314
50	Epinephrine with benzoate	6	27	70	142	191
50	” ” succinate	4	15	45	112	153
	0	18	62	128	214	316
10	Hydroquinone with benzoate	6	10	21	42	75
10	” ” succinate	17	61	111	189	285
10	p-aminophenol with benzoate	10	37	78	138	216
10	” ” succinate	8	12	17	32	41

compound. The rate of benzoate oxidation was an index of the amount of enzyme formation.

Results. Both catechol and protocatechuic acid have been identified as intermediates in the oxidation of benzoate by *Pseudomonas fluorescens*(4) and by certain mycobacteria (5). Both were oxidized slowly by our strain of *Pseudomonas,* and one or both were formed during benzoate oxidation as shown by the addition of 5-amino uracil which condenses with them to form a yellow compound. It was therefore rather surprising to find that catechol added with benzoate markedly inhibited the formation of the benzoic acid enzyme, and this effect was unchanged after 2 recrystallizations of the catechol. On the other hand, small amounts of catechol added at zero time with succinate stimulated enzyme formation as shown by decreased latent period and increased rate of benzoate oxidation. This effect might be described as a type of back adaptation. Protocatechuate, whether added at zero time or with benzoate, only stimulated enzyme formation. In order to test the possible effect of the carboxyl group, 3,4 dihydroxyphenalanine (dopa) was compared to epinephrine. When added with benzoate the former was 4-5 times as effective as an inhibitor. Thus a carboxyl group on the side chain does not neutralize the effect of the rest of the molecule. When dopa was added at zero time it had little effect, whereas epinephrine was almost equally active when added at zero time and with benzoate. These results are shown in Table I.

The percentage inhibition by catechol and dopa was independent of the amount of succinate. The oxidation rate of benzoate when it was added after 0.3 mg of succinate had been oxidized was for a time 3 times that after 0.1 mg of succinate, yet during this period the percentage inhibition varied only between 84 and 90 for both compounds at both concentrations of succinate. It is thus improbable that they are reacting directly with the precursors to make them unavailable for enzyme formation. The percentage inhibition by both compounds was also independent of the amount of benzoate when it was varied from 0.1 to 1.0 mg. Therefore mass action effects are not directly involved.

Other quinones were also active. The results with hydroquinone and p-aminophenol are shown in Table I. It is obvious that the latter was much more effective when added with succinate which indicates that it inhibited the formation of precursors more than their utilization. But hydroquinone like dopa had little effect when added with succinate which suggests that both compounds were rapidly inactivated in the presence of the bacteria oxidizing succinate and much less rapidly inactivated after the succinate oxidation was completed. It is obvious that all these compounds would readily autoxidize at pH 7.8 to quinones, and quinone added as such has exactly the same effect as hydroquinone. Comparison of naphthoquinones with anthraquinones showed that the former were more effective inhibitors in concentrations which had no effect on succinate oxidation and that they behaved like hydroquinone.

Discussion. Quinones readily inhibit enzyme formation. Catechol even though an intermediate in benzoate oxidation still behaves like other quinones until the enzyme for its oxidation is formed. If 10 γ/ml of catechol is added with succinate, then not only is some benzoate enzyme formed but also some catechol enzyme, and under these conditions the subsequent addition of 50 γ/ml of catechol with the benzoate causes little or no inhibition. At present there is no obvious reason why in the orthoquinone series dopa is the most active inhibitor or why p-aminophenol behaves differently from hydroquinone.

Summary. Catechol added with benzoate inhibits the formation of the enzyme which oxidizes benzoate in a strain of *Pseudomonas aeruginosa.* Added with succinate and ammonium sulfate 2 hours before benzoate it facilitates the formation of the benzoate enzyme. Protocatechuic acid only facilitates the formation. Dopa and epinephrine also inhibit enzyme formation, the former only when added with benzoate, the latter when added before or with benzoate. Hydroquinone, quinone, and p-aminophenol inhibit formation, the first 2 only when added with benzoate. The last is more effective when added before benzoate.

1. Bernheim, F., and DeTurk, W. E., *J. Bact.*, 1953, v65, 65.
2. Bernheim, F., *J. Pharmacol.*, 1954, v110, 115.
3. DeTurk, W. E., *Fed. Proc.*, 1953, v12.
4. Sleeper, B. P., and Stanier, R. Y., *J. Bact.*, 1950, v59, 117.
5. Gale, G. R., *J. Bact.*, 1952, v64, 131.

Received March 8, 1954. P.S.E.B.M., 1954, v85.

Effects of a Lysine Deficiency Upon Enzyme Activity in Rat Liver.* (20946)

J. W. BOTHWELL AND J. N. WILLIAMS, JR.
From the Department of Biochemistry, College of Agriculture, University of Wisconsin, Madison.

Several laboratories(1-4) have reported that liver xanthine oxidase activity is reduced in rats fed low protein diets. It has been noted by Richert and Westerfeld(5) that livers of weanling rats on a low protein ration (8% casein) lose about 50% of their liver choline oxidase activity. Williams and Elvehjem(6) have shown that with a tryptophan deficiency in the adult rat liver xanthine oxidase activity and endogenous respiration are reduced markedly, and the activity of succinic oxidase is decreased to some extent. Williams, Denton, and Elvehjem(7) have reported that in weanling rats the activity of rat liver xanthine oxidase can be completely removed by a methionine deficiency while succinic oxidase activity can be reduced somewhat. Unexpected results were obtained in some recent studies with a histidine deficiency (8) in which it was found that the absence of dietary histidine had no demonstrable effect upon xanthine oxidase, choline oxidase, or succinic oxidase, although weanling animals forcibly fed the deficient ration began to die

---

* Published with the approval of the Director of the Wisconsin Agricultural Experiment Station. Supported in part by a grant from the Nutrition Foundation, N. Y., and by a contract between the Regents of the University of Wisconsin and the Office of Naval Research, Washington, D. C.

after about 10 days.

In the present paper we wish to present results of the effects of a lysine deficiency upon liver enzymes. It was felt that deficiency of lysine, an amino acid which once deaminated cannot be reaminated, might show drastic effects on liver enzymes, effects which would be possibly comparable to those obtained with a non-protein ration. The present studies were conducted in a manner similar to those reported earlier(7,8) in which results of *ad libitum* feeding were compared with those of forcible feeding by stomach tube. These feeding procedures were compared since it is known that animals receiving an amino acid-deficient ration *ad libitum* show a marked loss in appetite(9). Thus it was considered imperative to keep food intake at a constant level to rule out any complications due to inanition. Also this makes it possible to compare the results directly with those obtained with a methionine or histidine deficiency, which have been previously reported(7,8). The enzyme systems chosen for study were liver xanthine oxidase, succinic oxidase, choline oxidase, and endogenous respiration. These systems are representative in that xanthine oxidase is a soluble cytoplasmic enzyme while succinic and choline oxidase are mitochondrial systems. Endogenous respiration may be considered a measure of general oxidative processes in the liver homogenate.

Procedure. Male weanling rats of the Holtzman strain were divided into 4 groups and placed on the purified ration of Ramasarma *et al.*(10) for one week or until an average weight of 55-60 g was reached. This ration consisted, in brief, of purified amino acid mix (16%), Salts IV(11), corn oil, the known vitamins including B_{12} (20 γ/kilo of ration) and sucrose. Two of the groups were fed *ad libitum* and 2 groups were fed forcibly. The latter groups were fed by stomach tube to insure a constant food intake. In previous similar experiments it was shown that weanling rats consume, on the average, 6 g of the synthetic ration per day. Therefore, this level was chosen to be fed. Feedings were made 3 times daily at 6-hour intervals. All groups were fed the complete purified ration for one week to accustom the animals to feeding procedures and to make sure that the animals were growing well at the beginning of the experiment. All animals were given water *ad libitum*. The results reported in this paper are the combined results of 3 replicate experiments. The animals were sacrificed for the enzyme determinations between 10 and 14 days when animals from the group forcibly fed the lysine deficient ration showed marked deficiency symptoms and appeared near death. Control animals were also removed for analysis when deficient animals were analyzed. The animals were killed by decapitation and the livers removed and placed immediately in cracked ice. A portion of each liver was quickly weighed, homogenized in 5 volumes of 0.039 M sodium potassium phosphate buffer, pH 7.3, and strained through gauze. This homogenate was used for xanthine oxidase determination according to the method of Axelrod and Elvehjem(12) and choline oxidase by the method of Williams *et al.*(13). A portion of the homogenate was diluted with buffer to give a 5% homogenate which was employed for measuring succinic oxidase according to the method of Schneider and Potter(14). Endogenous respiration was calculated from the oxygen uptake of the control flasks in the xanthine oxidase assay during the first 10-minute interval following the temperature equilibration period. Succinic oxidase, choline oxidase and endogenous respiration were assayed at 30°C while xanthine oxidase was determined at 37°C. This higher temperature was found necessary since erratic results have occasionally been obtained at 30°C(15). Nitrogen of the homogenates was determined by a semi-micro Kjeldahl procedure in duplicate.

Results. The enzyme activities in Table I are based on liver nitrogen. Xanthine oxidase activity is expressed in microliters of oxygen uptake per hour per g of liver nitrogen. Choline oxidase, succinic oxidase, and endogenous respiration activities are reported as microliters of oxygen uptake per hour per mg of liver nitrogen.

As seen in Table I, a lysine deficiency exerts a marked effect upon the activities of xanthine oxidase, succinic oxidase, and choline oxidase. A reduction to 58-76% of basal

TABLE I. Effects of Lysine Deficiency upon Liver Enzyme Activities and Liver Nitrogen of Rats Fed Forcibly and *Ad libitum.*

		Forcibly fed rats			*Ad libitum* fed rats		
Enzyme determined	Ration	No. of rats	Enzyme activity based on liver N.[*]	Ratio of deficient to control	No. of rats	Enzyme activity based on liver N.	Ratio of deficient to control
Xanthine oxidase	Basal	7	5030 ± 113[†]	.62	6	4800 ± 430	.57
	" -lysine	9	3120 ± 357		6	2780 ± 260	
Succinic oxidase	Basal	14	717 ± 59	.71	12	790 ± 51	.71
	" -lysine	15	510 ± 47		9	560 ± 49	
Choline oxidase	Basal	10	59 ± 6	.76	9	64 ± 8	.67
	" -lysine	13	45 ± 4		10	43 ± 4	
Endogenous respiration	Basal	13	94 ± 8	(.86)	10	69 ± 9	(1.11)
	" -lysine	15	81 ± 8		10	77 ± 5	
			mg N./g fresh liver			mg N./g fresh liver	
Liver nitrogen	Basal		25.8 ± .9	.87		25.8 ± .7	.88
	" -lysine		22.4 ± .3			22.6 ± .7	

[*] See text for method of expressing enzyme activities. Activity in terms of fresh liver wt can be calculated from enzyme results and figures for N./g fresh liver.

[†] Stand. error of mean.

activity was noted for these enzymes in terms of liver nitrogen. The cytoplasmic enzyme, xanthine oxidase, was somewhat more greatly affected than the mitochondrial enzymes, succinic oxidase, and choline oxidase. Endogenous respiration was not significantly altered by the lysine deficiency.

It can be noted that the reduction of activity of the enzymes studied was essentially the same whether the lysine deficient ration was fed forcibly or fed *ad libitum.* This was true even though the animals fed *ad libitum* showed a significantly lower food intake.

Nitrogen analyses of the livers of the rats indicate that a lysine deficient ration either fed *ad libtum* or fed forcibly causes a small but significant reduction in the nitrogen content of the liver. It should be noted, however, that the loss of enzyme activity is much greater than the loss of general liver nitrogen. Since a non-protein ration has been shown to reduce drastically the activity of xanthine oxidase in as short a period as 5 days(3), it is conceivable that a deficiency of any of the essential amino acids might have a similar effect. In the absence of one critical amino acid other amino acids would not be expected to be utilized for protein synthesis. Earlier results from this laboratory(7,8) have indicated that xanthine oxidase activity is reduced almost completely by a methionine deficiency while a histidine deficiency exerts no demonstrable effect upon this enzyme. As now observed, the lack of lysine exerts an intermediate effect in that the activity of xanthine oxidase is reduced to somewhat over half that of the normal.

The observed decrease in activity of succinic oxidase is greater than that produced by either of the other 2 amino acids (methionine and histidine) and is comparable to the decrease noted for both xanthine oxidase and choline oxidase. It can be seen that the lysine deficiency produces similar depression of activity in each system while histidine and methionine each produce a completely different pattern. Endogenous respiration has not been shown to be significantly changed by a deficiency of any of the 3 amino acids.

These results suggest that after 10 days to 2 weeks of forcibly feeding a ration devoid of lysine, although the animals are beginning to die, there appear to be sufficient body stores of lysine to maintain these enzymes to a moderate extent when a normal level of other essential and non-essential amino acids are fed. This is in contrast to the drastic reduction in activity obtained with a non-protein ration.

With the 3 amino acids studied thus far, no great differences have been found between the groups receiving the ration *ad libitum* or by stomach tube although in all cases animals receiving the deficient ration by stomach tube have begun to die before the end of the 2-week period. Therefore, it would appear that when fed a ration deficient in an essential amino acid the young animals used in these experiments are unable to cope with normal quantities of food intake. Thus they begin to die after about 10 days when fed a constant amount of food by stomach tube. Animals of similar weight and age fed *ad libitum*, however, appear to adjust to some extent to the deficient ration by reducing their food intake and consequently survive for longer periods of time.

Summary. 1. A lysine-free ration fed to weanling rats either *ad libitum* or by a forcible feeding procedure reduces the activity of liver xanthine oxidase, succinic oxidase, and choline oxidase to somewhat over half that of normal. 2. A lysine deficiency has little effect on liver endogenous respiration. 3. The deficiency produces an entirely different pattern of liver enzyme activity from that produced by a methionine or histidine deficiency.

---

1. Miller, L. L., *J. Biol. Chem.*, 1948, v172, 113.
2. Westerfeld, W. W., and Richert, D. A., *Fed. Proc.*, 1949, v8, 265.
3. Litwack, G., Williams, J. N., Jr., Feigelson, P., and Elvehjem, C. A., *J. Biol. Chem.*, 1950, v187, 605.
4. Williams, J. N., Jr., Feigelson, P., and Elvehjem, C. A., *ibid.*, 1950, v185, 887.
5. Richert, D. A., and Westerfeld, W. W., *ibid.*, 1952, v199, 829.
6. Williams, J. N., Jr., and Elvehjem, C. A., *ibid.*, 1950, v183, 539.
7. Williams, J. N., Jr., Denton, A. E., and Elvehjem, C. A., PROC. SOC. EXP. BIOL. AND MED., 1949, v72, 386.
8. Bothwell, J. W., and Williams, J. N., Jr., *J. Biol. Chem.*, 1951, v191, 129.
9. Frazier, L. E., Wissler, C. H., Steffee, R. L., Woolridge, R. L., and Cannon, P. R., *J. Nutrition*, 1947, v33, 65.
10. Ramasarma, G. B., Henderson, L. M., and Elvehjem, C. A., *ibid.*, 1949, v38, 177.
11. Hegsted, D. M., Mills, R. C., Elvehjem, C. A., and Hart, E. B., *J. Biol. Chem.*, 1941, v138, 459.
12. Axelrod, A. E., and Elvehjem, C. A., *ibid.*, 1941, v140, 725.
13. Williams, J. N., Jr., Litwack, G., and Elvehjem, C. A., *ibid.*, 1951, v192, 73.
14. Umbreit, *et al.*, Manometric techniques and related methods for the study of tissue metabolism, Minneapolis, 1945.
15. Litwack, G., Bothwell, J. W., Williams, J. N., Jr., and Elvehjem, C. A., *J. Biol. Chem.*, 1953, v200, 303.

Received March 8, 1954. P.S.E.B.M., 1954, v85.

Nutritional Studies with the Guinea Pig. B-Vitamins other than Pantothenic Acid.* (20947)

MARY ELIZABETH REID.

From the Laboratory of Biochemistry and Nutrition, National Institutes of Health, Public Health Service, U. S. Department of Health, Education and Welfare, Bethesda, Md.

The only B-vitamins for which there is unequivocal proof of dietary essentiality for life in the guinea pig are thiamine(1-3), folic acid(4-7), and pantothenic acid(8). There is considerable indication that a dietary supply of niacin also is essential(9,10).

A semi-synthetic diet recently developed for guinea pigs(11) has proven useful in evaluating the essentiality of several of the B-vitamins for these animals. In the present investigations deficiencies of thiamine, choline, folic acid, pyridoxine, niacin, and riboflavin have been demonstrated in the guinea pig, some for the first time. This report is concerned chiefly with a brief description of the gross deficiency symptoms and the effects of the deficiency on the growth rate and survival time. The effect of lack of a dietary supply of para-aminobenzoic acid, inositol, biotin, and vit. B_{12} has also been studied. Supplements of these substances were found

---

* Part of the data in this paper was presented at the American Institute of Nutrition in Chicago, April 1953 (*Fed. Proc.* 1953, v12, 472.)

TABLE I. Summary of Results with B-Vitamins other than Pantothenic Acid.

	Control group		Deficient guinea pigs				
Vitamin	No. of animals	Avg wt (g) 42nd day	No. of animals	Avg wt (g) of survivors 42nd day	No. of survivors	Avg survival time (days) of animals which died and day of 1st death	Deficiency symptoms, if any, other than retarded growth, anorexia, and weakness
Thiamine	28	333 ± 8*	20	209	1	26 (17)	Footnote [1]
Choline	18	334 ± 6	36	165 ± 10*	10	24 in 4 exp. (16, 21, 13, 18).† In other exp. all living with avg wt 181 g	” [2]
Folic acid	32	343 ± 5	32	281 ± 8	6	27 (22, 26, 16, 25)†	” [3]
Pyridoxine	12	339 ± 9	12	214 ± 6	7	28 (18, 29)	” [4]
Niacin	22	338 ± 8	23	245 ± 13	15	29 (27)	” [5]
Riboflavin	12	341 ± 15	12	284 ± 20	7	25 (18)	” [6]
PABA	13	336 ± 9	13	328 ± 11	13	—	No symptoms
Inositol	20	340 ± 8	20	311 ± 13	19	—	” ”
Biotin	18	341 ± 7	18	328 ± 9	17	—	” ”
B_{12}	22	346 ± 6	22	328 ± 10	22	—	” ”

* Stand. error. † Day of first death in different experiments.

[1] Emaciation, tremor, and spasm.

[2] Clear fluid frequently found in body cavities, kidneys pale and atrophic, some with scarred surface but no acute hemorrhage. No visible fattiness of liver.

[3] Decrease in No. of erythrocytes and leucocytes, an almost completely aplastic condition of bone marrow in animals which died early, tendency to fatty infiltration of liver, and hemorrhagic adrenals.

[4] Enlargement of adrenals, atrophy of sex organs, disturbances in gastrointestinal tract such as hemorrhagic condition of cecum.

[5] Drooling, paleness of feet, nose, and ears. Soiled coats.

[6] Paleness of feet, nose, and ears. Rough coats.

to be non-essential under our experimental conditions.

Procedure. Animals of the Hartley strain, 2 to 4 days old, with equal numbers of each sex and weight ranges of 95 to 115 g, were reared in screen-bottomed cages and were fed the basal semi-synthetic diet previously described(11). For study of the effects of a vitamin deficiency, the particular vitamin chosen for a study was omitted from the diet and the appearance, growth, and survival of the animals at the end of 6 weeks were compared with those of a group which had been maintained on the complete diet. In most of the experiments there were 6 to 8 animals in a group.

Results. The total number of animals employed in all the control groups was 96 and their average weight on the 42nd day was 339 g. Only one death occurred in the control animals. The means of the weights for the control groups in different experiments were very similar and are indicative of a consistent growth-rate on the control diet. The absence from the diet of thiamine, choline, folic acid, or pyridoxine caused pronounced deficiency symptoms. Table I shows that the weight gain of all the deficient groups was less than that of the controls and that the survival after 6 weeks was much less, particularly with respect to thiamine, choline, and folic acid. The data in the table show that a retardation in growth occurs also in the absence of a dietary supply of niacin or riboflavin, and that a considerable number of the animals succumbed to the deficiency as compared to almost 100% survival in the control groups. Some of the survivors in each of these 2 groups grew fairly well, however, and it appeared that some of them might have survived for an indefinite time. No specific symptoms characteristic of niacin and riboflavin deficiencies, such as dermatitis, were observed. In niacin deficiency there was no swelling of the tongue or reddening of the mouth, and in riboflavin deficiency there was no evidence of corneal opacity or vascularization.

No significant differences were found between the weights of the groups lacking a dietary supply of PABA, inositol, biotin, or vitamin B_{12} and their respective control groups. In the early phases of these studies the diet contained 3 mg of folic acid per kg. During this period the addition of PABA appeared to have beneficial results on growth but, in the later studies in which the content of folic acid was increased to 10 mg, there was no longer a growth response to PABA (see table, also reference 6). The results herein shown suggest strongly that under the present experimental conditions no additional inositol, biotin, or vit. B_{12} is essential for the maintenance of life in the guinea pig. Since, however, the weights of the control groups were slightly, though not significantly,† higher than those of the groups not supplied with the vitamins, it would be necessary to conduct further studies to obtain positive proof that these vitamins are not necessary for maximum growth. Mention should be made of the fact that Hogan and Hamilton(12) observed that some benefit resulted from the addition of inositol to purified diets for guinea pigs.

Discussion. There may be unidentified factors needed by the guinea pig which, if present, would improve the growth, but thus far we have not succeeded in demonstrating any benefit from feeding supplements of fresh kale (6 g daily of the green mesophyll tissue) or addition of a forage juice factor(13). Possibly these supplements would improve the reproductive performance but thus far, in most cases, the studies have been continued only through the first 6 to 8 weeks of growth. The data herein presented demonstrate, however, the need of the guinea pig for certain of the B-vitamins and afford the first clear-cut demonstration of a requirement of a dietary supply of choline, riboflavin, and pyridoxine. The guinea pig appears to be similar to the chick in its requirement for many of the B-vitamins, including a high requirement for folic acid. In of both of these animals folic acid deficiency symptoms are produced in very young animals by the absence of the vitamin from the diet without resorting to the use of anti-metabolites. The guinea pig's requirement of a dietary supply of certain B-vitamins is quite unlike that of the rabbit (14). This is somewhat surprising since both animals subsist naturally on green forage. The practice employed in the present experiments of removing the guinea pigs from the mother when only 2 to 4 days old may have influenced the bacterial flora of the gastrointestinal tract, thereby indirectly affecting the vitamin requirements. As compared to rabbits (left with the mother 21 days or more), this difference in post-natal time spent with the mother, together with apparently a greater tendency to coprophagy in the rabbit (15), may have an important bearing on the dietary requirement of B-vitamins in the 2 types of animals.

B-vitamin studies with the guinea pig are being continued using the basal diet herein employed and a determination of the quantitative requirement, if any, of each of these vitamins is being made.

Summary. A determination has been made of the essentiality or non-essentiality of vitamins of the B group as components of a semi-synthetic diet developed especially for guinea pigs. Clear-cut deficiencies of choline, pyridoxine, and riboflavin were produced for the first time. In addition, a dietary need of thiamine, niacin, and folic acid has been confirmed. It appears that in the presence of an adequate supply of folic acid, PABA is not essential in the diet. Under our conditions no requirement has yet been demonstrated for inositol, biotin, or vit. B_{12}.

1. Carlström, B., and Jonsson, C., *Skand. Vet.*, 1939, v29, 181; *Nutrition Absts. and Rev.*, 1940, v9, 71.

2. Lilleengen, K., *Skand. Vet. Tidskr.*, 1940, v30, 165; *Biol. Abst.*, 1946, v20, 92.

3. Pellegrino, J., and Pinho, J., *Rev. brasil. biol.*, 1946, v5, 61; *Biol. Abst.*, 1946, v20, 285.

4. Woolley, D. W., and Sprince, H., *J. Biol. Chem.*, 1945, v157, 447.

5. Mannering, C. J., and Brown, R. A., Unpublished, 1946. Quoted by Mannering, Vitamins and Hormones, 1949, VII, 208.

6. Woodruff, C. W., Clark, S. L., Jr., and Bridgeforth, E. B., *J. Nutrition*, 1953, v51, 23.

7. Reid, M. E., *Fed. Proc.*, 1953, v12, 472.

8. Reid, M. E., and Briggs, G. M., *J. Nutrition*, 1954, v52, April.

---

† According to the T-test of Fisher.

9. Harris, L. J., *Chemistry and Industry,* 1939, v58, 471.

10. Cannon, M. D., Mannering, C. J., Elvehjem, C. A., and Hart, E. B., PROC. SOC. EXP. BIOL. AND MED., 1946, v63, 414.

11. Reid, M. E., and Briggs, G. M., *J. Nutrition,* 1953, v51, 341.

12. Hogan, A. G., and Hamilton, J. W., *ibid.,* 1942, v23, 533.

13. Kohler, G. O., Elvehjem, C. A., and Hart, E. B., *ibid.,* 1938, v15, 445.

14. Olcese, O., Pearson, P. B., and Schweigert, B. S., *ibid.,* 1948, v35, 577.

15. Kulwich, R., Struglia, L., and Pearson, P. B., *ibid.,* 1953, v49, 639.

Received March 11, 1954. P.S.E.B.M., 1954, v85.

Autoradiographical Distribution and Excretion Studies with S^{35}-Labelled Penicillin. (20948)

SVEN ULLBERG. (Introduced by W. P. Boger.)

From the Department of Pharmacology, Royal Veterinary College, Stockholm, Sweden.

Previous investigations of the pharmacology of penicillin have depended in general on bacteriological methods of penicillin estimation. In the present investigation autoradiography has made possible a more detailed localization of the radioactive substances in histological sections, so that the distribution and fate of penicillin in normal mice could be studied. Some preliminary observations have also been made on the distribution of penicillin in diseased tissue, and on the inhibitory effect of probenecid* on the excretion of penicillin by the kidneys.

Methods. Radioactive (S^{35}-labelled) penicillin was prepared biosynthetically according to methods similar to those described by Smith and Hockenhull(1) but using stirred bottles and aeration. Crystalline sodium penicillin was obtained via the ethylpiperidine salt. Paper chromatography showed almost entirely benzylpenicillin. The histological technic was complicated by the solubility of penicillin in water and fixation fluids. The labelled penicillin was injected intravenously in a tail vein. A dose of one millicurie per g of body weight was employed, which corresponds to 8 μg of penicillin per g of body weight. Animals were killed at different times after injection. In most cases the entire body of the mouse was rapidly frozen in isopenthane at —170°C. The frozen mice were mounted on large stages and allowed to reach —10°C, when they were sectioned. All of the sections were dried at —10°C. In some of the mice small pieces were taken from organs and treated by the usual freeze-drying methods with subsequent paraffin-embedding and sectioning at room temperature. Contact autoradiographic technic was used with exposure for some weeks in iron presses at —10°C.

Results. Distribution in normal tissues. Penicillin is rapidly distributed to the tissues from the blood stream. In all cases the relative distribution of penicillin concentration in the various tissues was similar from 5 minutes after the injection until the penicillin began to disappear from the tissues.

Fig. 1 shows the distribution of penicillin in an adult mouse 20 minutes after injection. The numbers indicate the relative degrees of radioactivity in the organs as a percentage of that of the blood. These were obtained by measuring on a dried section with a mica-window G-M tube, when the whole section, except for the measured area, was covered.

The greatest penicillin concentration is found in the kidneys, which are responsible for the elimination of most of the penicillin, but the liver is not to be disregarded as an excretory organ, as can be seen from the high activity in the biliary system. All the activity in the lumen of the intestine appears to come from the biliary system. The subcutis

---

* Probenecid is the generic name for *p*-(di-n-propylsulfamyl)-benzoic acid supplied under the trademark 'Benemid' (U.S.A.) and 'Probecid' (Sweden).

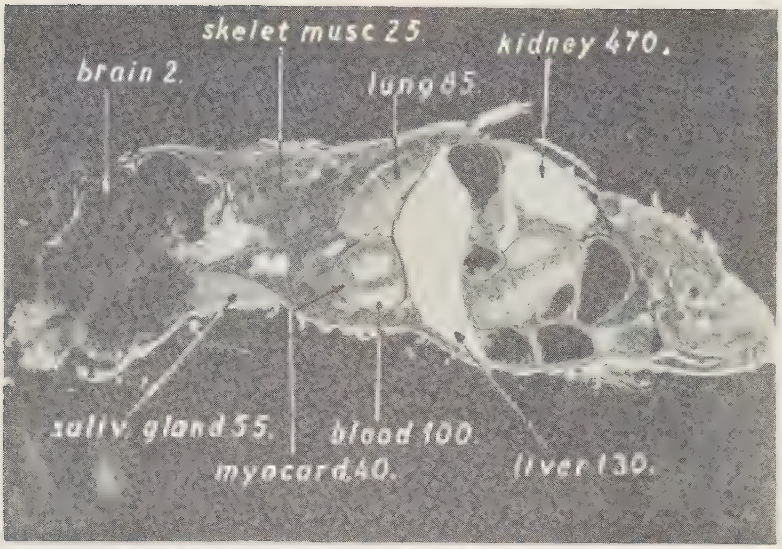


FIG. 1. Autoradiograph showing the distribution of labelled penicillin in an adult mouse 20 min. after intravenous injection. The numbers refer to the penicillin concentration as percentage of the blood concentration.

and the lungs show a high concentration of penicillin, while it is markedly lower in skeletal muscle. The penicillin concentration in compact bone is low, in the brain very low, and still lower in the cerebrospinal fluid.

Fig. 2 (autoradiograph of a pregnant mouse 30 minutes after an intravenous injection) shows that the activity is high in the uterine wall, rather low in the fetus, and almost nonexistent in fetal fluids. In addition, it appears that the fetal brain contains essentially less penicillin than the other parts of the fetus, indicating that the blood-brain barrier is functioning at this early stage.

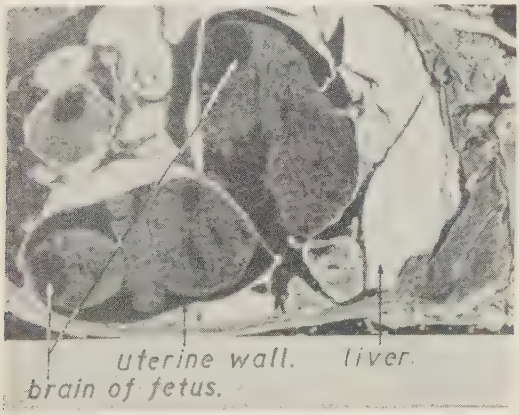


FIG. 2. Autoradiograph showing the uterus with two fetuses in a pregnant mouse. White areas correspond to high penicillin concentration.

Fate of penicillin in body. After 1½-2 hours most of the penicillin is excreted, but some activity is still present in the kidneys, bladder, liver, and intestines. Penicillin also disappears rather slowly from the cerebrospinal region and the eyes. Rowlands *et al.* (2) measured the excretion of intramuscularly administered radioactive penicillin in the urine of anesthetized cats. They showed a recovery in the urine of 100% by measurement of the radioactivity with a G-M tube. A large correction for self-absorption in the samples was necessary. The present investigations on mice were based on the opposite principle. The penicillin remaining in the body, and not that excreted in the urinefi was measured. The results are in agreement with those of Rowlands *et al.* in that neither penicillin nor its possible breakdown products remain in the tissues. On the other hand, my results show that some radioactivity terminates in the alimentary tract, despite the parenteral administration of the penicillin in mice, which is at variance with Rowlands' results in cats.

Fig. 3 shows an autoradiograph of a mouse 5 hours after intravenous injection. It is obvious that radioactivity has disappeared

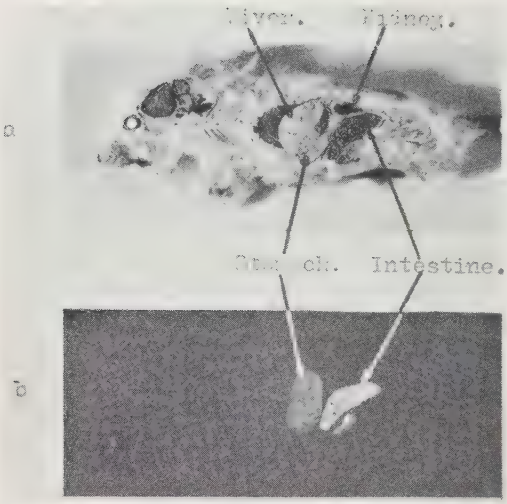


FIG. 3. a) Photograph of the section surface of a mouse killed 5 hr after intravenous injection of labelled penicillin. b) Autoradiograph of (a).

from all tissues, even the kidneys and liver, while the activity excreted via the bile ducts to the intestines is not reabsorbed to any large extent but remains in the intestinal lumen. There is also some activity in the stomach, which could have emanated from the penicillin excreted in the saliva. (This autoradiograph was produced by bringing the even surface of the frozen block into direct contact with the film. As S^{35} produces rays of only a short range, pictures of relatively good resolution could be produced in this simple manner.) Throughout the experiments intravenous injections have been used to obtain rapid distribution and to avoid any inactivation of penicillin during resorption from an intramuscular injection site.

Beyer(3) in his monograph on penicillin pharmacology states: "Inactivation of penicillin in the body is of uncertain significance in the overall elimination of the compound." According to most of the information available, up to 100% of parenterally administered penicillin is excreted in a still biologically active state. In view of this, it does not appear probable that inactivation in the body can seriously alter the distribution picture in a study of this kind. If a physico-chemical separation of possible breakdown products from intact labelled penicillin in body fluids and tissue extracts could be carried out, it might be possible to give a more definite answer to the question of inactivation.

Pathological tissues. Tissue changes due to infectious agents can, to a great extent, affect the ability of penicillin to reach the bacteria. Fig. 4 shows the distribution of penicillin 30 minutes after injection in the case of a subcutaneous abscess of natural origin. The contents of the abscess were of caseous character. The penicillin has apparently passed through the abscess membrane but has not reached the center. (The concentration of penicillin in the subcutis is always relatively high.) The experimental animal received probenecid before the injection of penicillin.

Site of action of probenecid. There are a number of compounds that block the renal excretion of penicillin on a competitive basis. Probenecid is at present the most promising of these agents. Renal clearance tests(4) have shown that probenecid can reduce penicillin excretion in the urine to about 20% of normal, which is interpreted as inhibition of the tubular secretion while glomerular filtration continues as usual.

Fig. 5 shows an autoradiograph of the

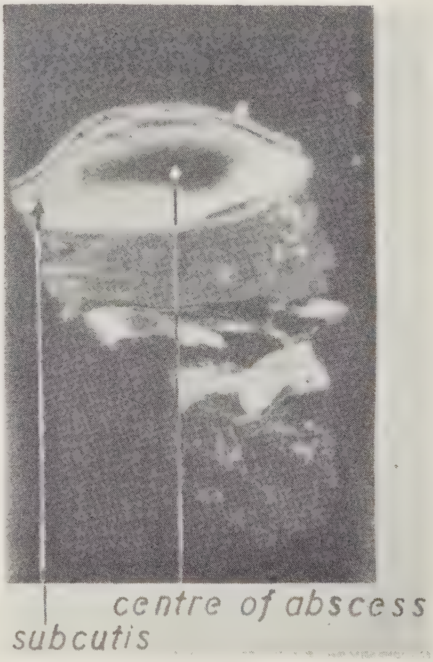


FIG. 4. Autoradiograph showing the ability of penicillin to pass an abscess membrane.

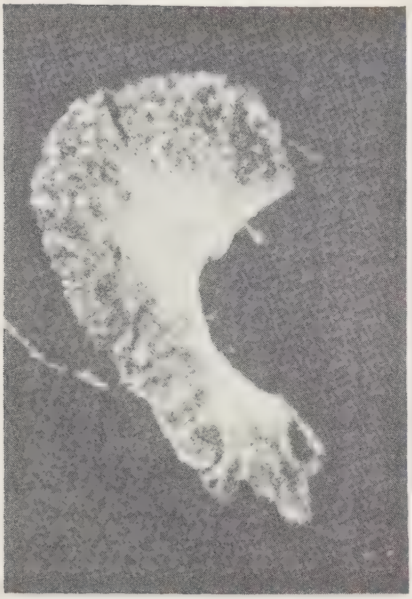


FIG. 5. Autoradiograph of a kidney of a mouse that received probenecid before labelled penicillin.

kidney of a mouse that received 0.2 mg of probenecid per g weight *per os* 2 hours before the injection of penicillin. The glomeruli are visible against a background of relatively penicillin-free tubuli, while in the case of animals that received only penicillin the renal tubuli show a very high degree of activity.

Summary. Radioactive S^{35}-labelled penicillin has been injected into mice and its distribution in the various tissues of the body studied by autoradiographical technics. The results correlate well with the observations that have been made using the microbiological assay for penicillin. In the kidneys the concentration is always, and in the liver generally, higher than in the blood, but in other organs during the whole course of distribution and excretion it is lower than in the blood. In the lungs the concentration nearly approximates that of the blood, while in most tissues it is considerably lower. It is lowest in the central nervous system. The method here described can be applied to the study of penicillin distribution in diseased tissues. Preliminary observations have shown the diffusion of labelled penicillin into abscesses, and the difference in distribution of penicillin in the kidneys under the influence of probenecid and after the administration of penicillin alone.

1. Smith, E. L., and Hockenhull, D. J. D., *J. appl. Chem.*, 1952, v2, 287.

2. Rowlands, S., Rowley, D., and Stewart, H. C., *Lancet*, 1948, v2, 493.

3. Beyer, K. H., *Pharmacological Basis of Penicillin Therapy,* Charles C. Thomas, Publisher, Springfield, Ill., 1950, p42.

4. Beyer, K. H., Russo, H. F., Tillson, E. K., Miller, A. K., Verwey, W. F., and Gass, S. R., *Am. J. Physiol.*, 1951, v166, 625.

Received March 11, 1954. P.S.E.B.M., 1954, v85.

Plasma Lipoprotein Changes Following Thermal Injury in the Dog.* (20949)

RICHARD S. THOMAS, BURTON E. VAUGHAN, ELAINE L. WALKER, AND NELLO PACE.
From the Department of Physiology, School of Medicine, and Office of Naval Research Unit No. 1, University of California, Berkeley.

Following burn injury, there is consistently a rise in the α-globulin fraction of plasma in human beings, dogs, and various other animals, as shown by electrophoretic studies (1-4). The fact that, in dogs, the α-globulin fraction contains all of the plasma lipoproteins(5,6) suggested to us that these lipoproteins might be considerably altered by thermal injury. Further support for such a relationship comes from chemical fractionation studies(7).

In the present investigation, the lipoproteins were isolated from the plasma of burned dogs and characterized by flotation in an analytical ultracentrifuge. Marked changes in the lipoprotein fractions were shown to

---

* These studies aided by contract between Office of Naval Research, Department of the Navy, and the University of California.

occur as a consequence of thermal injury.

Methods. Five dogs were maintained for at least 2 weeks prior to the experiment on a standard diet of dog biscuit, and were deprived of food for 12 hours immediately preceding the experiment. The dogs were anesthetized with nembutal and burned by a hot water scald. Three animals were immersed for 30 seconds to the axillae in water at 70°C. The hind legs of a fourth dog were immersed in water at 80°C for 30 seconds. A fifth dog, a control, was not burned but was otherwise treated similarly to the other animals, *i.e.*, anesthetized with nembutal, and then bled at intervals and deprived of food for 72 hours. The control factors, therefore, included anesthesia, the fasting of the experimental dogs due to loss of appetite after injury, and blood loss due to sample collecting. Samples of blood, 20-30 ml in volume, were drawn into oxalated tubes immediately preceding burning and at several intervals up to 72 hours afterward. A biuret determination of total plasma protein and a hematocrit determination were made, and the high and low density lipoproteins were separately isolated from each plasma sample. The low density lipoproteins were isolated and characterized by a technic similar to that of Gofman *et al.*(8). Seven ml of plasma were diluted with 2 ml of an approximately 3.4 M potassium bromide solution, which was standardized to give a resultant protein-free solution density of 1.063, and the preparation was centrifuged at 130,000 x g for 12 hours in a Spinco Type C rotor. The top one ml in the centrifuge tube, containing the low density lipoproteins, which floated, was pipetted off for examination in the analytical ultracentrifuge. The high density lipoproteins were isolated from the bottom fraction of the low density preparation. This bottom fraction, which contained the proteins that sedimented in a medium of density 1.063, was thoroughly mixed and a 4 ml sample removed. To this sample were added 5 ml of an approximately 4.1 M potassium bromide solution, such that the new protein-free solution density was 1.21. This preparation, similar to that of Lewis and coworkers(5), was centrifuged at 130,000 x g for 24 hours and the top one ml, containing the high density lipoprotein, was removed for ultracentrifugal analysis. As can be seen from volume considerations, the low density lipoprotein was concentrated 7-fold and the high density lipoprotein by a factor of 3.5 over the original concentration in the plasma.[†] The increased resolution of components of the low density lipoprotein during the analytical run, made possible by higher concentration and lower solvent density, was the principal reason for isolating the low density lipoprotein separately from the high density lipoprotein. The analytical runs were made in a Spinco model E ultracentrifuge at 260,000 x g at 25-26°C. The low density lipoproteins, in a solvent of density 1.063, were characterized by their S_f values, in accordance with the technic and nomenclature of Gofman and co-workers(8). The high density molecules, in a solvent of density 1.21, were characterized by —S values following Lewis *et al.*(5). Two-channel cells, similar to those described by Milch (9), were used in most of the runs. These cells allow the base line to be directly superimposed on the lipoprotein schlieren pattern. Lipoprotein concentrations were calculated from the areas of pattern tracings, using the value 0.00154 $(g/100\ cc)^{-1}$ for the specific refractive increment.

Results. Table I indicates the changes from pre-burn values of the plasma concentrations of high and low density lipoprotein and total (biuret) protein. It also shows the relative distribution of lipoprotein in various S_f or —S classes for each pre- and post-burn sample. The S_f or —S classes were arbitrarily chosen to best show the changes in distribution.

It will be seen that 2 major changes common to all 4 experimental animals following thermal injury are a shift of the high density lipoprotein to faster flotation rates and a marked increase in the concentration of the low density lipoprotein. The normal high density lipoprotein consisted, in all dogs studied, principally of a single component with —S 3, and a smaller component, often unresolved, at —S 6. Following thermal in-

---

[†] A few plasma samples were less than 7 ml. These were made to volume with isotonic sodium chloride solution.

TABLE I. Concentration and Distribution in Flotation Rate Classes of Plasma Lipoprotein from 4 Dogs with Thermal Injury and a Control Dog.

	Absolute values of pre-burn protein conc. (g/100 cc)				% of pre-burn protein concentrations			% distribution of lipoprotein in each sample‖						
								High D L (−S class)			Low D L (S_f class)			
Dog	High D* L	Low D L	Total plasma protein	Time after thermal injury (hr)	High D L	Low D L	Total plasma protein	0-4	4-8	8-14	0-5	5-10	10-25	25-50
A†	.48	.06	—	Before	100	100	—	71	25	4	—	—	—	—
				4.5	100	200	—	56	38	6	—	—	—	—
				7.5	100	170	—	50	44	6	—	—	—	—
				25	130	220	—	34	51	15	—	—	—	—
				30	110	190	—	33	51	16	—	—	—	—
B‡	.72	.08	6.7	Before	100	100	100	88	12	0	75	25	0	0
				6	89	130	87	84	16	0	76	24	0	0
				23	—	140	90	71	22	5	66	34	0	0
				50	110	250	93	60	34	6	80	17	3	0
C‡	.54	.23	6.1	Before	100	100	100	62	36	2	73	25	2	0
				6	120	120	102	68	29	3	71	23	6	0
				25	130	160	100	51	43	6	44	38	17	1
				48	—	190	100	33	54	13	23	34	35	8
D‡	.58	—	6.2	Before	100	100	100	88	12	0	33	56	11	0
				25	120	170	103	57	36	7	20	36	44	0
				48	110	240	98	51	39	10	27	59	14	0
				71	94	—	103	68	22	10	—	—	—	—
E§	.57	—	6.2	Before	100	100	100	85	15	0	51	43	6	0
				25	110	70	103	88	12	0	53	47	0	0
				47	86	90	97	87	13	0	49	44	7	0
				71	100	100	103	88	12	0	68	29	3	0

* D = density; L = lipoprotein.

† Hind legs immersed in water at 80°C for 30 sec.

‡ Immersed to axillae in water at 70°C for 30 sec.

§ Control dog.

‖ Total concentration of high or low density lipoprotein in each sample is taken to be 100%.

jury, the principal normal component slowly decreased, while a polydisperse distribution of components, with flotation rates up to —S 12-13, increased. There appears to be no consistent trend of total concentration changes in the high density lipoprotein of the experimental dogs, although there is considerable fluctuation in both the experimental and control values. Apparently the decrease in the normal component roughly equaled the increase in the new components. This is illustrated in Fig. 1a-1b. These changes appear to be reversible. In one dog, D, which recovered from the thermal injury, the distribution started to shift back toward normal by 71 hours.

The low density lipoprotein in unburned dogs was present in rather low concentration compared to that of the high density lipoprotein, and consisted of a polydisperse group of components of S_f 1-10 with the largest components at S_f 3-5. After thermal injury, however, the concentration of the low density molecules approximately doubled. This increase is not ascribable to a hemoconcentration effect because the total plasma protein values remained essentially constant. Furthermore, in dog B, the total plasma protein concentration actually decreased while the low density lipoprotein concentration increased.

A variety of distribution pattern changes for the low density lipoprotein were observed in the 4 dogs following thermal injury, as may be seen in Fig. 1d-h. In dog B (Fig. 1d-f), a slight increase in faster components, S_f 5-10, occurred at 23 hours, but by 50 hours a large increase in a slow component, S_f 4, had occurred. In dog C (Fig. 1g-h), a large increase of faster components occurred and progressed continuously. The distribution changes seen in dog A were somewhat similar

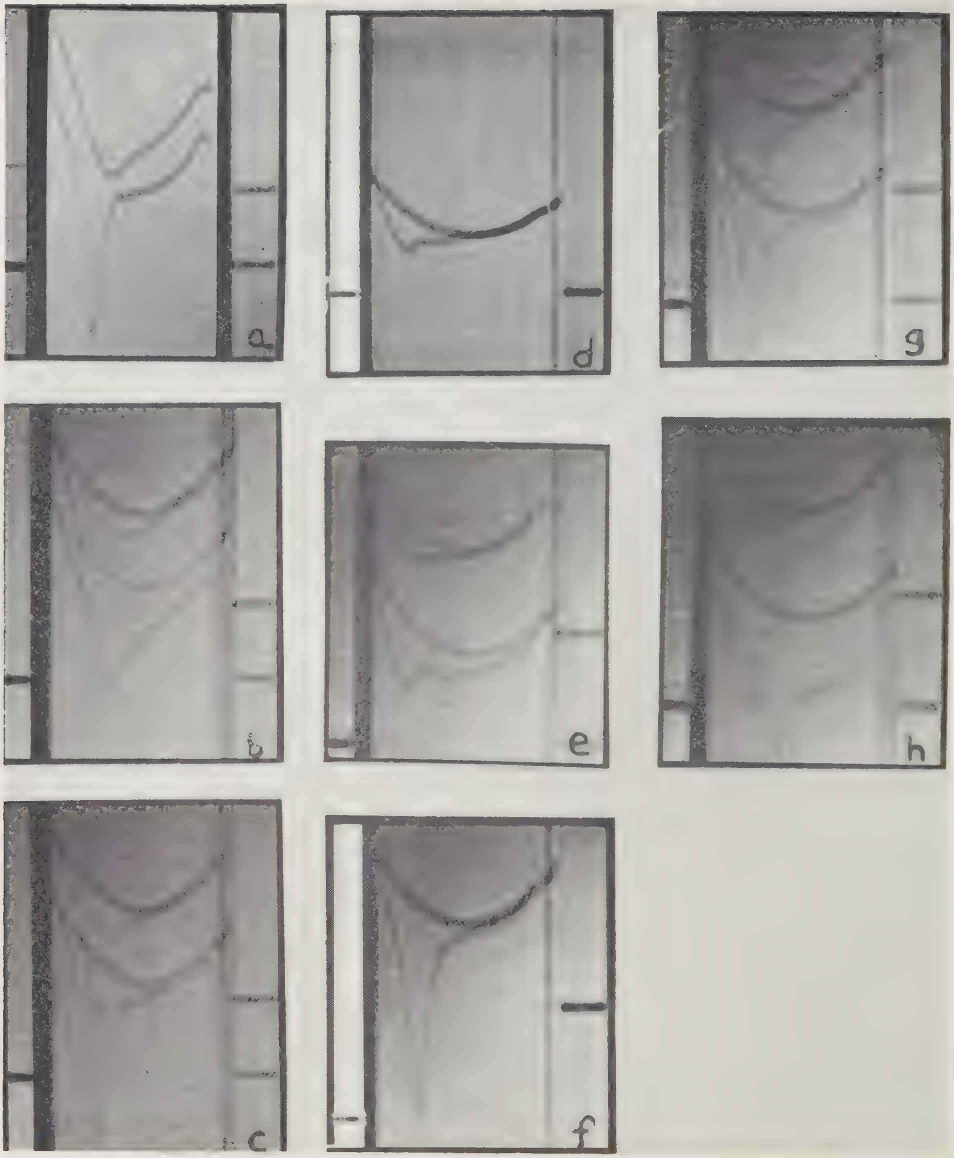


FIG. 1. Ultracentrifuge patterns of plasma lipoprotein from dogs before and after thermal injury.

a. High density lipoprotein from dog A. Upper pattern, 30 hr after burn; lower pattern, before burn. Conc. 4× from plasma.

b. High density lipoprotein from dog D. Upper pattern, before burn; lower pattern, 48 hr after burn. Conc. 3.5× from plasma.

c. High density lipoprotein from dog E (unburned control). Upper pattern, 71 hr after initial sample; conc. 2.5× from plasma. Lower pattern, initial sample; conc. 3.5× from plasma.

d. Low density lipoprotein from dog B, before burn. Conc. 7× from plasma.

e. Low density lipoprotein from dog B. Upper pattern, 6 hr after burn; conc. 4× from plasma. Lower pattern, 23 hr after burn; conc. 7× from plasma.

f. Low density lipoprotein from dog B, 50 hr after burn. Conc. 7× from plasma.

g. Low density lipoprotein from dog C. Upper pattern, before burn; lower pattern, 6 hr after burn. Conc. 7× from plasma.

h. Low density lipoprotein from dog C. Upper pattern, 25 hr after burn; lower pattern, 48 hr after burn. Conc. 7× from plasma.

Boundaries are moving from right to left in above frames. All runs made at 260000 × g at 25-26°C. a-c taken 64 min. after full speed at a bar angle of 60°. d-h taken 32 min. after full speed at a bar angle of 50°, except for frame d where the bar angle was 60°.

to those of dog C.‡ In dog D, the changes were intermediate between those of dogs A and C in that both a pronounced shift to faster components and an increase in a slow component occurred.

Essentially no changes were seen in either the high or low density lipoproteins of the control dog. Although there was some fluctuation in the total concentration of both the high and low density lipoprotein, there was no specific trend. The distribution of both types of lipoprotein remained completely unchanged. The high density lipoprotein pattern of this dog is shown in Fig. 1c.

Discussion. While these results must be considered preliminary owing to the small number of dogs studied and to technical difficulties which produced gaps in the data, they are sufficient to show that substantial changes do occur in the plasma lipoproteins of the dog following thermal injury.

The increase in concentration of the low density lipoproteins is probably metabolically related to the shift of the high density lipoprotein to faster flotation rates. However, this increase in concentration of the low density molecules is probably not due simply to the inclusion of rapidly floating high density lipoprotein in the low density lipoprotein fraction. The 2 classes of molecules appear to remain distinct, as indicated in the experiment with dog A. Here, the high and low density lipoproteins were isolated and analyzed together at density 1.21. The preburn distribution of components consisted of components —S 2-7 and —S 23-27. The latter components correspond to the components S_f 1-10 in a medium of density 1.063. After thermal injury the distribution shifted respectively to —S 2-13 and —S 23-40 but there were no components in the range of —S 13-23.

It is difficult to speculate on the significance of these changes since the metabolic mechanisms of plasma lipoprotein regulation are rather obscure at present. However, low density lipoprotein changes similar to the ones reported here have been observed in rabbits subjected to local cold injury(10,11) and total body irradiation(12). The changes probably represent a response of the animal to tissue injury in general, just as do the α-globulin changes which have approximately the same time course and can be produced by many different kinds of injury(1).

The lipoprotein changes are probably not associated directly with the shock syndrome, and appear to occur later. In this investigation, furthermore, the conditions of thermal injury to the dogs were such that they did not go into severe shock. Although the hematocrit increased significantly a few hours after thermal injury, it was back to normal at 24 hours when the lipoprotein changes began to appear, at which time some of the dogs were quite active.

Summary. Plasma lipoproteins of 4 dogs were studied by flotation in the ultracentrifuge before and after thermal injury. After injury, the high density lipoprotein of all 4 shifted to higher flotation rates without consistent changes in concentration. The low density lipoprotein increased in concentration in all 4 dogs with variable flotation rate changes among the 4. No significant changes were seen in the lipoproteins of a control dog. It is suggested that these lipoprotein changes contribute to the electrophoretic α-globulin changes seen generally after injury.

---

‡ The distributions of low density lipoprotein in samples from dog A are not listed in the table since this low density lipoprotein was run at a density of 1.21 and thus is not comparable quantitatively with the low density lipoprotein of the other dogs.

1. Shedlovsky, T., and Scudder, J., *J. Exp. Med.*, 1942, v75, 119.
2. Chanutin, A., and Gjessing, E. C., *J. Biol. Chem.*, 1946, v165, 421.
3. Gjessing, E. C., and Chanutin, A., *ibid.*, 1947, v169, 657.
4. Perlmann, G. E., Glenn, W. W. L., and Kaufman, D., *J. Clin. Invest.*, 1942, v22, 627.
5. Lewis, L. A., Green, A. A., and Page, I. H., *Am. J. Physiol.*, 1952, v171, 391.
6. Zeldis, L. J., Alling, E. L., McCoord, A. B., and Kulka, J. P., *J. Exp. Med.*, 1945, v82, 411.
7. Gjessing, E. C., Ludewig, S., and Chanutin, A., *J. Biol. Chem.*, 1947, v170, 551.
8. Gofman, J. W., Lindgren, F., Elliott, H., Mantz, W., Hewitt, J., Strisower, B., and Herring, V., *Science*, 1950, v111, 166.
9. Milch, L. J., *Lab. Invest.*, 1953, v2, 441.
10. Milch, L. J., Redmond, R. F., and Calhoun,

W. W., *Am. J. Med. Sci.*, 1953, v225, 416.

11. Redmond, R. F., Milch, L. J., and Calhoun, B. S., *J. Lab. Clin. Med.*, 1953, v42, 1112.

12. Hewitt, J. E., Hayes, T. L., Gofman, J. W., Jones, H. B., and Pierce, F. T., *Cardiologia*, 1952, v21, 353.

Received March 12, 1954. P.S.E.B.M., 1954, v85.

Recovery of a Rickettsia of the Spotted Fever Group from *Microtus pennsylvanicus* from Virginia. (20950)

DOUGLAS J. GOULD AND MARIE L. MIESSE.* (Introduced by Robert Traub.)

From the Department of Entomology and Department of Virus and Rickettsial Diseases, Army Medical Service Graduate School, Walter Reed Army Medical Center, Washington, D. C.

Certain of the small mammals native to North America, particularly rodents and rabbits, have been suspected of serving as reservoirs of Rocky Mountain spotted fever because (a) they are the natural hosts of the tick vectors of this disease; (b) many are experimentally susceptible to infection with spotted fever; and (c) antibodies against spotted fever rickettsiae have been demonstrated in sera from some of these small mammals. However, despite the fact that the disease has been long studied in the United States, no confirmed strains of spotted fever have yet been reported isolated from wild-caught mammals in this country.

During 1952 and 1953 isolation studies were undertaken at the Army Medical Service Graduate School in an effort to detect the presence of spotted fever rickettsiae among small wild mammals collected in known endemic areas in the vicinity of the District of Columbia. The present paper reports the isolation of a strain of rickettsiae belonging to the spotted-fever group from the tissues of a meadow mouse, *Microtus pennsylvanicus*, collected during this study from a suburb of Alexandria, Va.

Materials and methods. One hundred and five small mammals were collected from yards and fields believed to be sources of recent cases of spotted fever in the vicinity of Alexandria, Va., and of Gaithersburg and Laurel, Md. These animals were trapped alive by means of small metal box traps and were returned to the laboratory for rickettsial isolation studies.

Ten percent suspensions of the brain, spleen and liver from each of the wild-caught animals were prepared in sterile saline and inoculated intraperitoneally into male guinea pigs. A portion of the inoculum in each case was streaked on blood agar plates to determine the presence of contaminating bacteria. The test guinea pigs were examined daily for fever (rectal temperature of 104°F or higher) and signs of disease. Those guinea pigs which became febrile 3 or more days following inoculation were routinely sacrificed on the second or third day of fever and transfer of suspensions of their liver, spleen, testes and tunica vaginalis were made intraperitoneally to fresh guinea pigs and mice and into the yolk sacs of 7-day-old embryonated eggs. Impression smears were prepared from the uncut surfaces of spleen and liver and from scrapings of the tunica vaginalis of passage guinea pigs. These preparations were stained by Giemsa's and Macchiavello's methods and examined microscopically for the presence of intracytoplasmic rickettsia-like bodies. When an infectious agent suggestive of rickettsiae was isolated in guinea pigs, convalescent sera drawn from these animals 21 to 30 days after the subsidence of fever were tested for complement-fixing antibodies against spotted fever rickettsiae. Complement-fixation tests were performed according to the methods employed in the Department of Virus and Rickettsial Diseases of the Army Medical Service Graduate School. Sera from passage guinea pigs were titrated in the presence of specific

---

* The authors gratefully acknowledge the technical assistance of Edward Michelitch and Georgina Lancaster.

washed spotted fever and rickettsialpox antigens which were prepared from the yolk sacs of embryonated eggs infected with the Bitterroot strain of *Rickettsia rickettsii* and the MK strain of *R. akari,* respectively, according to the method of Plotz *et al.*(1). Guinea pigs which had recovered from infection with a suspected rickettsial agent were subjected to challenge inoculations with Bitterroot or Azain strains of *R. rickettsii.* (The Azain strain was isolated from a fatal human case of spotted fever at the AMSGS in 1943, while the Bitterroot strain was obtained from the Rocky Mountain Laboratory in Montana).

Results. Rickettsial isolation attempts were made from the following animals during the course of this study: 65 meadow mice (*Microtus pennsylvanicus*), 17 white-footed mice (*Peromyscus leucopus*), 17 house mice (*Mus musculus*), 4 short-tailed shrews (*Blarina brevicauda*), one weasel (*Mustela frenata*), and one jumping mouse (*Zapus hudsonius*). Tissues from only one of the 105 wild caught animals contained a demonstrable rickettsial agent; the guinea pigs inoculated with tissues from the other 104 animals remained afebrile, showed no signs of disease and failed to develop complement-fixing antibodies against spotted fever.

A strain of rickettsiae, subsequently identified as belonging to the spotted fever group, was isolated from the tissues of an apparently healthy *Microtus* (No. B14009) trapped on June 18, 1952 near Alexandria, Va. Three guinea pigs, each inoculated intraperitoneally with one ml of a suspension of brain, spleen and liver from this animal, developed fevers of 105°F on the fifth day following inoculation. One of the sick guinea pigs was killed on the third day of fever and a suspension of its spleen, liver and testes was inoculated intraperitoneally into normal guinea pigs. The *Microtus* agent was maintained through more than 15 passages in guinea pigs. The passage materials were bacteriologically sterile.

The characteristic disease induced in guinea pigs by the *Microtus* agent had an incubation period of 2 to 8 days and a febrile phase of 5 to 9 days duration but was not lethal. The agent produced no scrotal reactions in early passage guinea pigs, but in later passages some animals developed swelling and erythema of the scrotum. However, the necrosis and sloughing of superficial scrotal tissues, ears and foot pads characteristically produced in guinea pigs by the Bitterroot strain of *R. rickettsii* were not observed in animals infected with the *Microtus* agent.

Postmortem examination of infected guinea pigs revealed splenomegaly, inguinal lymphadenopathy and hyperemia of the testes and tunica vaginalis. Rickettsiae were extremely scarce in smears prepared from tissues of early passage guinea pigs, but were seen occasionally within the cytoplasm of cells in smears from scrapings of the tunica vaginalis of later passage animals.

The *Microtus* agent was lethal for chick embryos 4 to 6 days following inoculation, and bacilliform rickettsiae were seen in smears from the yolk sacs of infected eggs. Known infectious material failed to produce obvious disease in white mice during 2 serial passages in that host.

Convalescent sera from guinea pigs which had been infected with the *Microtus* agent fixed complement in the presence of specific Rocky Mountain spotted fever antigen at titers of from 1/40 to 1/160, but these same sera failed to fix complement in the presence of rickettsialpox specific antigen.

Guinea pigs recovered from infection with the *Microtus* agent resisted infection with the Azain and Bitterroot strains of *R. rickettsii.* Similarly, Azain-immune guinea pigs were resistant to overt infection with the *Microtus* agent. Results of the cross-immunity tests are given in Table I.

Discussion. The recovery of rickettsiae of the spotted fever group from a wild mammal is of special interest from the standpoint of the ecology of Rocky Mountain spotted fever in this country. Although naturally infected mammals have never been definitely demonstrated in the United States, strains of spotted fever have been isolated from wild-caught opossums (*Didelphis aurita, D. paraguayensis,* and *D. marsupialis*) in Brazil(2,3). Brazilian workers(3) have also reported dogs, rabbits (*Sylvilagus minensis*) and cavies (*Cavia aperea*) to be susceptible to experimental infection with spotted fever. However, since

TABLE I. Relation of *Microtus* Agent to *R. rickettsii*: Cross-Immunity Tests in Guinea Pigs.

		Results of challenge				
		Fever		Scrotal reaction		
Challenge strain	Guinea pig group	No. G.P. with	Avg days duration	No. G.P. with	Intensity*	No. G.P. survived/No. tested
RMSF (Azain strain)	*Microtus* agent Immune	0/2	0	0/2	0	2/2
	Normal	2/2	10	2/2	4+	2/2
RMSF (Bitterroot strain)	*Microtus* agent Immune	0/2	0	0/2	0	2/2
	Normal	2/2	10	2/2	4+	1/2
Microtus agent	Azain immune	0/2	0	0/2	0	2/2
	Normal	2/2	8	0/2	0	2/2

* Scrotal reaction intensity: 1+ to 2+ = swelling and erythema; 3+ = swelling, erythema and adhesion of testicles; 4+ = necrosis and sloughing of scrotal skin plus swelling, erythema and adhesion of testicles.

quite different species of ticks and mammalian hosts are involved, the ecology of spotted fever in South America may differ greatly from that which exists in North America.

In the United States, Jellison(4) has suggested that the cottontail rabbit *Sylvilagus nuttallii,* may be an important reservoir of spotted fever. The distribution of this rabbit coincides roughly with that of the disease in the western United States, and wild cottontails are important hosts of the Rocky Mountain wood tick, *Dermacentor andersoni,* which is the principal vector of the disease in the West. Protection tests carried out by Parker(5) suggested the occurrence of spotted fever infections in wild rabbits and rodents. Complement-fixing antibodies against spotted fever rickettsiae have been demonstrated by Parker and his coworkers(6) in the sera of wild-caught cottontails and snowshoe rabbits (*Lepus americanus*) in Montana, and these authors also isolated strains of spotted fever from rabbit ticks, *Haemaphysalis leporis-palustris,* collected from cottontails in the same region. Jellison(4) and Parker *et al.* (7) have cited the probable isolation of the agent of spotted fever by Hassler and his coworkers from a pocket gopher (*Geomys breviceps dutcheri*) trapped in Oklahoma. Unfortunately, the agent isolated from the gopher was lost before its identity was established. Shepard and Topping(8) reported the presence of complement-fixing antibodies in the sera of dogs from several known endemic regions of the United States. Miller (9) found that experimentally infected foxes and raccoons showed no visible signs of disease, but they did respond by the formation of complement-fixing antibodies against *R. rickettsii.*

Seven of the *Microtus* used in this study, including the one positive animal, had one or more nymphs of *Dermacentor variabilis* attached to them when collected. This tick is the principal vector of spotted fever in the eastern half of the United States. While the adults of *variabilis* frequently attack man, this stage is more often found on dogs, livestock, deer and wild carnivores. The larval and nymphal stages parasitize smaller mammals, particularly rodents. All stages of this tick are able to transmit spotted fever, and the infection can be transferred from stage to stage and to successive generations through the eggs of infected female ticks(10). The eastern meadow mouse, *Microtus pennsylvanicus,* is one of the most common hosts of the immature stages of *D. variabilis* where the range of the tick and meadow mouse coincide(11). The distribution of *M. pennsylvanicus* and its subspecies, according to Hall and Cockrum(12), extends over most of Canada and the northern half of the United States from the Atlantic seaboard as far west as British Columbia and northeastern Washington, and it is found as far south as Georgia and northern New Mexico. Its distribution coincides to a great extent with that of *D. variabilis* in the eastern United States and extends into the range of *D. andersoni* in the Rocky Mountain region. Jellison(13) found the meadow mouse highly susceptible to experimental infection with spotted fever, and he was able to demonstrate tick-to-mouse-to-tick transfer of the infection in this host by the simultaneous feeding of infected nymphs and non-infected larvae of *D. andersoni* on the same animal. The present paper provides the first direct evidence of the occurrence of spotted fever infections in meadow mice in nature. However, it should be emphasized that the findings of this study do not necessarily give an indication of the actual prevalence of spotted fever infections among wild meadow mice, or for that matter, among the other species of mammals dealt with in this study. Furthermore, the role played by these mammals in the maintenance of spotted fever in nature remains to be determined.

Summary. The recovery of a strain of rickettsia belonging to the spotted fever group, presumably *Rickettsia rickettsii*, from the tissues of a wild meadow mouse, *Microtus pennsylvanicus*, is described. The evidence supporting its identification as *R. rickettsii* is presented and the implications of these findings are discussed.

1. Plotz, H.. Reagan, R. L., and Wertman, K., Proc. Soc. Exp. Biol. and Med., 1944, v55, 173.
2. Travassos, J., *Compt. rend. Soc. biol.*, 1937, v126, 1054.
3. ———, *Proc. 4th Int. Cong. Trop. Med. Malaria*, 1948, vI, 414.
4. Jellison, W. L., *Pub. Hlth. Rep.*, 1945, v60, 958.
5. Parker, R. R., *Arch. Path.*, 1933, v15, 398.
6. Parker, R. R., Pickens, E. G., Lackman, D. B., Bell, E. J., and Thraikill, F. B., *Publ. Hlth. Rep.*, 1951, v66, 455.
7. Parker, R. R., Kohls, G. M., and Steinhaus, E. A., *ibid.*, 1943, v58, 721.
8. Shepard, C. C., and Topping, N. H., *J. Infect. Dis.*, 1946, v78, 63.
9. Miller, J. K., *Ann. Int. Med.*, 1950, v33, 1398.
10. Parker, R. R., Philip, C. B., and Jellison, W. L., *Am. J. Trop. Med.*, 1933, v13, 341.
11. Smith, C. N., Cole, M. M., and Gouck, H. K., *U. S. Dept. Agric. Tech. Bull.*, 1946, No. 905.
12. Hall, E. R., and Cockrum, E. L., Univ. Kansas Publ., *Mus. Nat. Hist.*, 1953, v5, 373.
13. Jellison, W. L., *Pub. Hlth. Rep.*, 1934, v49, 363.

Received March 15, 1954. P.S.E.B.M., 1954, v85.

Skin Sterols IV. Distribution of Δ^7-Cholestenol.* (20951)

W. L. Miller, Jr. and C. A. Baumann.

From the Department of Biochemistry, College of Agriculture, University of Wisconsin, Madison.

Δ^7-cholestenol, 7-dehydrocholesterol, and certain other sterols react much more rapidly in the Liebermann-Burchard test than cholesterol itself, and this difference in rate has been made the basis for a determination of total "fast-acting" sterols(1,2). 7-dehydrocholesterol, which occurs in the skins of most species examined(3), can be measured spectrophotometrically and the concentration of Δ^7-cholestenol and related substances calculated by difference. Commercial cholesterol contains 0.62% of the Δ^7 sterol, and the results of a selenium test suggest that sterols from certain other sources may contain as much as 4.34%(4). Much larger amounts of Δ^7-cholestenol, 23-25% of the total sterols, have been isolated from rat skins(5).

Experimental. Twenty-five to 500 g of fresh skin (single skin or pooled samples) were extracted with acetone and the sterols isolated as the digitonide by procedures previously outlined(5). Dry digitonide containing 50-500 μg of sterol was placed in a standard Evelyn tube and dissolved by warming with 2 ml of glacial acetic acid. After cooling, 4.2 ml of reagent (freshly prepared mixture of 20 ml of redistilled acetic anhydride and one ml of H_2SO_4 cooled to room temperature) was added and the color read in an Evelyn colorimeter at 1.5 and 38 minutes.

---

* Published with the approval of the Director of the Wis. Agric. Exper. Station. Presented in part before the Amer. Soc. of Biological Chemists, New York, April 15, 1952, (*Fed. Proc.*, 1952, v11, 261).

TABLE I. Fast-Acting Sterol in Sterols from Skins and Certain Other Materials.

Source of sterols	No. of animals	Total fast-acting sterol (%)†	7-Dehydrocholesterol (%)†
♂ Albino rat	5	36.3	.84
♀ " "	5	30.9	
♀ Wild "	1	32.9	
♂ Cotton "	8	30.6	.29
♂ C_3H mouse	10	21.2	1.20
♀ Guinea pig	14	12.7	.37
♂ Chinchilla	2	5.7	.14
♂ Rabbit	1	3.3	.24
♂ Pig	1	5.6	4.70
♂ Dog	3	2.1§	.28
Calf	1	1.8	
♀ Human*	1	1.5	.43
Human atheromatous aorta‡	1	0.3	
Chicken (4 wk old)	10	1.4	
♂ Cat	1	1.2	
Sheep	1	8.8	.19
Wool	1	15.2	.14
U.S.P. Lanolin		8.6	.12
Egg yolk	2 eggs	1.4	
Halibut liver oil		n‖	

* Normal skin from a mammary flap, supplied by Dr. W. H. Jaeschke, Wisconsin General Hospital.

† Numbers refer to percentages of fast-acting sterol or 7-dehydrocholesterol in sterol preparations isolated from various sources indicated.

‡ Supplied by Dr. H. J. van Baaren, Department of Pathology, University of Wisconsin Medical School.

§ Skin sterols from a fourth dog (adult female) contained 12.3% of fast-acting sterol with 0.28% of 7-dehydrocholesterol; this was not included in the average for dogs, since the other 3 samples analyzed yielded totally different values, *viz.*, 1.9, 1.8 and 2.7% of fast-acting sterol.

‖ n = negligible.

The concentrations of fast-acting sterols (as Δ^7-cholestenol) and of cholesterol were then calculated by means of the appropriate equations† $F = 0.197\ R_1$ and $S = 0.751\ R_2 - 0.367\ R_1$(1,2). For the determination of 7-dehydrocholesterol in certain of the samples the sterols were regenerated from the digitonides(6), crystallized from 80% ethanol, and the absorption at 2815 Å measured in ethanol solution in a Beckman spectrophotometer. Rats were fed an adequate semisynthetic diet or one deficient in pantothenic acid(7). Certain weanlings and adults were made hyperthyroid by feeding 0.25% of iodinated casein (Protamone) for 4 weeks, while others were made hypothyroid by feeding 0.1% of 6-n-propyl-2-thiouracil‡ for 8 days and 0.02% of the drug thereafter(8).

Results. Fast-acting sterols in skins of various species. In general sterols isolated from the skins of rodents contained the highest concentrations of the fast-acting sterols. Rat skin sterols contained about 35% calculated as Δ^7-cholestenol and only 1% or less of the preparation was 7-dehydrocholesterol. Sterols from most other rodents also contained substantial proportions of total fast-acting sterol (Table I). The fast-acting component of pig skin sterols was mainly 7-dehydrocholesterol. Sterols from sheep skin (not free of wool), wool, and lanolin gave a strong color at 1½ minutes but the final cholesterol color was a yellow-green rather than the characteristic green of cholesterol. It is therefore doubtful whether the color at 1½ minutes was due entirely to Δ^7-cholestenol. The triterpene alcohols of wool are reported to react with the Schoenheimer-Sperry reagent with maximum absorption at 458 mμ(9,10). Digitonides from sheep skin were low in 7-dehydrocholesterol. Skin sterols from most other species including man contained only small amounts of the fast-acting sterols, 1.2-1.8%. A sample of a human atheromatous aorta was practically devoid of the new sterol, as was halibut liver oil (Table I). Contrary to a previous report(4) eggs contained only low amounts.

Effect of age and sex. After 3 weeks of age the percentage of Δ^7-cholestenol in the sterols from the skin of male rats was significantly higher than that in females. In both sexes there was an increase in fast-acting sterol with age, most of the increase occurring prior to maturity. The concentration of 7-dehydrocholesterol on the other hand remained relatively constant. The percentage of fast-acting sterol also increased with age in the sterols of guinea pig skin (Table II), but was only about half that in rats at comparable ages.

---

† F and S refer to mgs. of the two types of sterols in 6.2 ml of reaction mixture and R_1 and R_2 are L units at 1.5 and 38 minutes respectively.

‡ Supplied by Calco Chemical Division, American Cyanamid Company.

TABLE II. Percentages of Fast-Acting Sterol in Skin Sterols from Rats and Guinea Pigs of Various Ages.

Species	Age (days)	No. of animals	Total fast-acting sterol (%)		7-Dehydrocholesterol (%)	
			♂	♀	♂	♀
Rat	2	17	9.5		1.13	
	21	17, 16	21.0	19.0		
	37	7, 6	34.9	22.2	1.52	1.43
	50	5		24.1		
	63	5	34.2			
	365	11, 11	38.9	23.0	1.02	0.98
Guinea pig	5	2	4.9		—	
	Adult	14	—	12.7	—	0.37

Localization, metabolic rate and pantothenic acid deficiency. In agreement with a previous observation(1) skin from the back contained more Δ^7-cholestenol and 7-dehydrocholesterol than the skin from the abdomen (Table III).

Rats deprived of pantothenic acid contained a normal percentage of fast-acting sterols in the skin, in spite of a substantial deficiency shown by an average weight of 112 g at 6 weeks compared to 278 g for control rats.

TABLE III. Fast-Acting Sterols of Rat Skin from Various Parts of the Body: Effects of Basal Metabolic Rate and of Pantothenic Acid Deficiency.

	Sex	Skin	No. of animals	Age	Total fast-acting sterol (%)	7-Dehydrocholesterol (%)
C*	♂	Back	4	1 yr	43.3	1.33
C	♂	Abdomen	4		31.1	.52
C	♀	Back	7		26.9	1.48
C	♀	Abdomen	7		19.3	.42
C	♂	Entire skin	5	3-8 mo	36.3	
He†	♂	"	4		38.2	
Ho‡	♂	"	4		41.0	
C	♀	"	5		30.9	
He†	♀	"	6		38.9	
Ho‡	♀	"	5		29.0	
C	♀	"	5	7 wk	24.1	
He†	♀	"	5		34.1	
Ho‡	♀	"	5		25.1	
C	♂	"	5	9 wk	34.2	
PA.d.	♂	"	8		35.3	

* C = control; He = hyperthyroid; Ho = hypothyroid; PA.d. = pantothenic acid deficient.

† Avg of 3.83 L of O_2 consumed/24 hr/dm^2—as compared to 1.6 L for normal rats.

‡ Avg of 1.03 L of O_2 consumed/24 hr/dm^2—as compared to 1.6 L for normal rats.

Alterations in basal metabolic rate falied to alter the percentage of fast-acting sterol in the skin of male rats, but in females there appeared to be an increase in the fast-acting component of the hyperthyroid animals (Table III).

Discussion. The very large amounts of Δ^7-cholestenol in the skin of rodents and its presence elsewhere in only trace amounts suggests that the sterol may have a particular function in rodent skin. One possibility is that it may be in equilibrium with 7-dehydrocholesterol, serving as a storage reservoir from which precursors of vit. D may be derived as needed. It would thus be of value to the rat to have more Δ^7-sterol in the skin of the back than in the abdomen, and the high amounts in the skin of the ear(1), where there is relatively little hair, would be particularly useful. An accessory function of the new sterol rather than an indispensible one is suggested by the fact that the skins of many species do not contain it in any substantial amount. Something more than storage is suggested by the constancy in the ratio Δ^7-cholestenol to cholesterol throughout adult life, since true storage substances, *e.g.*, hepatic vit. A, show no such constancy. Nor does the alleged relationship to vit. D explain why there should be more Δ^7-cholestenol in the skins of males than of females. The increase in fast-acting sterol in the skin of rats of both sexes prior to maturity parallels a similar change in the sebaceous secretions of the human scalp, where there is a marked increase in the concentration of the fungistatic fatty acids at puberty(11;12).

Since these experiments were completed

Langdon and Bloch have postulated that Δ^7-cholestenol may be an intermediate in the biosynthesis of cholesterol(13). If this should be true, the large amounts of Δ^7-cholestenol in rodent skin would imply a less effective conversion to cholesterol in this tissue than in the rest of the body or in the skins of other species.

Summary. 1. The concentration of Δ^7-cholestenol in rat skin sterols increased rapidly from birth to 5-6 weeks of age but remained fairly constant thereafter. Skin sterols from adult male rats contained from 35-40% of Δ^7-cholestenol while skin from females contained 25-30%. These proportions did not change in severe pantothenic acid deficiency nor in hypothyroidism. Hyperthyroidism, however, appeared to increase the concentration of Δ^7-cholestenol in sterols from female rats. 2. The total fast-acting sterol in the skins of the wild rat and cotton rat were similar to those of the laboratory rat, while sterols from the skins of the mouse and guinea pig contained about 15% of a fast-acting component. The amounts of Δ^7-cholestenol were low in the skins of the pig, rabbit, dog, calf, chicken, and cat. A sample of human skin and a human atheromatous aorta contained negligible amounts of the new sterol.

1. Moore, P. R., and Baumann, C. A., *J. Biol. Chem.*, 1952, v195, 615.

2. Idler, D. R., and Baumann, C. A., *ibid.*, 1953, v203, 389.

3. Windaus, A. and Bock, F., *Z. physiol. Chem.*, 1934, v245, 168.

4. Fieser, L. F., *J. Am. Chem. Soc.*, 1951, v73, 5007.

5. Idler, D. R., and Baumann, C. A., *J. Biol. Chem.*, 1952, v195, 623.

6. Bergmann, W., *ibid.*, 1940, v40, 471.

7. Lih, H., King, T. E., Higgins, H., Baumann, C. A., and Strong, F. M., *J. Nutr.*, 1951, v44, 361.

8. Miller, W. L., Jr., and Baumann, C. A., *Cancer Research*, 1951, v11, 634.

9. Lederer, E., and Tchen, P. K., *Bull. Soc. Chim. Biol.*, 1945, v27, 419.

10. Lederer, E., *J. Chem. Soc.*, 1949, 2115.

11. Rothman, S., Smiljanic, A. M., and Shapiro, A. L., PROC. SOC. EXP. BIOL. AND MED., 1945, v60, 394.

12. Weitkamp, A. W., Smiljanic, A. M., and Rothman, S., *J. Am. Chem. Soc.*, 1947, v69, 1936.

13. Langdon, R. G., and Bloch, K., *Fed. Proc.*, 1953, v12, 235.

Received March 15, 1954. P.S.E.B.M., 1954, v85.

Effect of 5-Bromouracil on Utilization of Thymidine by *Streptococcus faecalis*.* (20952)

WILLIAM H. PRUSOFF.† (Introduced by Arnold D. Welch.)

From the Departments of Pharmacology, Schools of Medicine, Western Reserve University, Cleveland, O., and Yale University, New Haven, Conn.

The introduction of a halogen in substitution for a hydrogen or an alkyl group in a metabolite not infrequently results in the formation of a compound with antimetabolite activity. 5-Bromouracil has been reported by Hitchings *et al.*(1) and by Weygand *et al.*(2) to inhibit the growth of *L. casei* and *S. faecalis*, respectively, in basal media supplemented with thymine. In addition, Hitchings *et al.* (1) have observed that 5-bromouracil, in the absence of thymine or folic acid, supported limited growth of *L. casei*. In view of evidence that thymine and its desoxyriboside are incorporated by different mechanisms in these microorganisms(3), studies have been made of the effect of 5-bromouracil on the growth of *S. faecalis* in a medium supplemented with either thymidine or thymine.

---

* This investigation was supported by a grant from the American Cancer Society, as recommended by the Committee on Growth, National Research Council, and in part by a grant from the National Institutes of Health, U. S. Public Health Service.

† The present address of the author is Department of Pharmacology, Yale University, School of Medicine, New Haven, Conn.

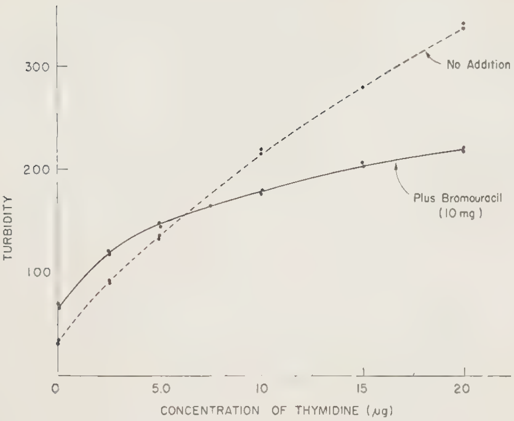


FIG. 1. The effect of 5-bromouracil on the growth of *S. faecalis* in a medium supplemented with thymidine.

Experimental. Studies with growing culture. The organism used in these studies was *Streptococcus faecalis* (ATCC 8043) which was grown in a standard basal medium(4). 5-Bromouracil‡ (10 mg) was placed in one of 2 series of assay tubes, to which increasing amounts of thymidine were added (final volume: 10 ml). The tubes were inoculated and then incubated for 22 hours at 37°C. Growth was measured as turbidity in a Klett-Summerson photoelectric colorimeter (Filter No. 66). The results are shown in Fig. 1. At this level, bromouracil significantly stimulated the growth of *S. faecalis* when thymidine was present in amounts of 5 μg or less per 10 ml of medium, but as the concentration of thymidine was increased, the inhibitory effect of bromouracil became evident; this was not abolished even by 300 μg of thymidine.

In a second type of experiment increasing quantities of 5-bromouracil were added to 2 series of tubes, one of which contained 10 μg of thymine and the other 20 μg of thymidine. They were inoculated and incubated at 37°C for 20 hours. The results are shown in Table I. In agreement with the investigations of Weygand *et al.*(2), 5-bromouracil was able completely to inhibit the growth of *S. faecalis* in the medium supplemented with thymine; however, when thymidine replaced thymine the pattern was changed remarkably, although definitely inhibitory, bromouracil depressed growth to only 50% of maximum, even at a concentration of 1%. There was no relationship between the method of addition of the halogenated pyrimidine to the medium and its effect on the growth of this organism. Bromouracil, when sterile-filtered and added aseptically to previously autoclaved media, or when autoclaved in the media, inhibited the growth of *S. faecalis* to the same extent.

Studies with resting cells. Medium (500 ml) supplemented with thymine (2.5 mg) was sterilized in one-liter Erlenmeyer flasks for 20 minutes at 15-lb pressure. After inoculation with *S. faecalis*, the flasks were incubated at 37°C for 20 hours. The cells were harvested by centrifugation and twice resuspended in saline and recentrifuged. Thymidine was incubated with the washed resting cell suspensions in the presence and absence of 5-bromouracil. The composition of the incubation mixtures (total volume, 1.0 ml) was as follows: thymidine, 1 μM; phosphate buffer, λ 0.5 M, 0.1 ml, pH 8.1; and wet bacterial cells, 50 mg, either in the presence or absence of 5-bromouracil, 25 μM. After an incubation period of 3 hours at 37°C the reaction was stopped by the addition of 0.1 ml 25% $HClO_4$(5). After cooling in an ice bath for 10 minutes the acidified suspensions were centrifuged in the cold and analyses for total desoxyribose by the Stumpf reaction(6), free pentose by the Nelson reaction as modified

TABLE I. Effect of 5-Bromouracil on Growth of *S. faecalis* in Media Supplemented with Thymine or Thymidine.*

Conc. of 5-bromouracil (mg)	Growth	
	Thymine (10 μg)	Thymidine (20 μg)
0	340	338
5	122	226
10	95	226
20	72	225
30	—	220
40	—	212
50	54	204
60	—	192
70	—	180
80	—	166
90	—	158
100	—	150

* Growth measured as turbidity in a Klett-Summerson colorimeter (filter #66).

---

‡ The author is grateful to Dr. W. L. Holmes and Dr. S. Greenbaum of this department for the preparation of 5-bromouracil.

by Manson and Lampen(7), and unreacted thymidine by microbiological assay with *Leuconostoc citrovorum* (ATCC 8081) were performed.

Either in the presence or absence of bromouracil, thymidine as such completely disappeared. Although all desoxyribose was lost in the absence of the halogenated pyrimidine, in its presence the desoxypentose was retained completely and in a bound form. These results strongly suggest that the desoxyriboside of 5 -bromouracil had been formed in a manner similar to the conversion of 6-azathymine to the corresponding desoxyriboside(8).

Discussion. 5-Bromouracil in an otherwise unsupplemented medium not only supports the growth of *S. faecalis* to a limited extent, but in addition is incorporated into the nucleic acid of this organism(11). Therefore, an increase in the amount of growth when bromouracil was added to media which contained suboptimum concentrations of thymidine, was expected. However, it was not anticipated that a non-competitive inhibition of the growth of *S. faecalis* would be caused by the analog even in the presence of very high concentrations of the normal metabolite, thymidine.

It would appear, therefore, that there are at least 2 pathways for thymidine utilization, and that the major one is not inhibited by bromouracil. Under conditions where the supply of thymidine is minimal, the nucleoside may be shunted to this major pathway exclusively, and hence bromouracil not only does not exert any inhibition, but is also utilized to some extent for growth. The overall effect is one of an increase in growth beyond that observed with thymidine alone. When the thymidine concentration is increased beyond minimal levels, a second or minor pathway may be utilized. If the minor pathway involves a cleavage of thymidine to free thymine, then the inhibition produced by bromouracil would be understandable. Although *S. faecalis* possesses a nucleosidase which could catalyze this reaction(8), evidence has been presented previously which indicates that the cleavage of thymidine to free thymine, probably is not the major pathway for thymidine utilization in this microorganism(3).

Evidence suggesting the formation of the desoxyriboside of 5-bromouracil with resting cell suspensions of *S. faecalis* has been presented. It is conceivable that this compound also may be formed during conditions of ordinary growth. Normally, the higher the ratio of bromouracil to thymidine, the larger would be the amount of the unnatural desoxyriboside formed. However, in the growth experiments, when the ratio of thymidine to bromouracil was increased, not only was there no reversal of inhibition, but there appeared to be an increase in the degree of antagonism. This would appear to eliminate 5-bromouracil-desoxyriboside as the causative agent in the observed inhibition. The corresponding riboside of bromouracil, 5-bromouridine, has been reported reversibly to inhibit the growth of Neurospora (No. 1298) in a medium supplemented with uridine or cytidine(9). However, 5-chlorouridine(9) and 5-iodouridine (10) have no effect on the growth of *S. faecalis* in media supplemented with folic acid or thymine and, in addition, the iodinated derivative exerted no inhibition of growth in the presence of thymidine. Investigations of the effect of halogenated derivatives of the *desoxyribosides* on the growth of organisms which have a thymidine requirement are indicated.

Summary. 1. Whereas 5-bromouracil competitively inhibited the growth of *S. faecalis* in a medium supplemented with thymine, an incomplete, and non-competitive inhibition was observed in the presence of thymidine. 2. 5-Bromouracil prevented the disappearance of desoxyribose when resting *S. faecalis* cells are incubated with thymidine, and this is interpreted as signifying the formation of the desoxyriboside of 5-bromouracil. 3. Possible pathways of thymidine metabolism in *S. faecalis* are discussed.

The author wishes to express his appreciation to Dr. Arnold D. Welch for his interest in this work and for advice concerning the preparation of the manuscript.

---

1. Hitchings, G. H., Falco, E. A., and Sherwood, M. B., *Science,* 1945, v102, 251.

2. Weygand, Von F., Wacker, A., and Grisebach, H., *Z. für Naturforch.,* 1951, v6b, 177.

3. Prusoff, W. H., Holmes, W. L., and Welch, A. D., Submitted for publication.

4. Lepper, H. A., Editor: *Official Methods of Analysis of the Association of Official Agricultural Chemists*, Washington, D. C., Seventh Edition, 1950, 784.

5. Hoffmann, C. E., Personal communication.

6. Stumpf, P. K., *J. Biol. Chem.*, 1947, v169, 367.

7. Manson, L. A., and Lampen, J. O., *ibid.*, 1951, v191, 95.

8. Prusoff, W. H., *Fed. Proc.*, 1953, v12, 358.

9. Fukuhara, T. K., and Visser, D. W., *J. Biol. Chem.*, 1951, v190, 95.

10. Prusoff, W. H., PROC. SOC. EXP. BIOL. AND MED., 1954, v85, 567.

11. Von Weygand, F., Wacker, A., Dellweg, H., *Z. für Naturforsch.*, 1952, v7b, 19.

Received March 15, 1954. P.S.E.B.M., 1954, v85.

Microbiological Activities of Iodoorotic Acid and Iodouridine.* (20953)

WILLIAM H. PRUSOFF.† (Introduced by Arnold D. Welch.)

From the Departments of Pharmacology, Schools of Medicine, Western Reserve University, Cleveland, O., and Yale University, New Haven, Conn.

In a previous paper it was shown that neither 5-iodoorotic acid-I^{131} nor 5-iodouridine-I^{131} is concentrated by the regenerating liver of the rat or by normal or certain neoplastic tissues of rats or mice(1). For several species of microorganisms, however, certain halogenated pyrimidines have been described as antimetabolites(2-4) or have exerted limited activity as growth factors(2). In fact, 5-bromouracil appears to be incorporated into the nucleic acids of *Streptococcus faecalis*(5), and to interfere markedly with the utilization not only of exogenous thymine, but also of a related compound formed endogenously in the presence of derivatives of folic acid(6). These results with bacteria suggested a reinvestigation of the effect of iodoorotic acid and iodouridine on the growth of certain microbial species.

Experimental. 5-Iodoorotic acid-I^{131} *and 5-iodouridine-*I^{131}. These compounds were synthesized by previously published methods (1). *Determination of radioactivity.* The concentration of I^{131} in the cells was determined with the aid of a well-type scintillation counter constructed by MacIntyre(7). Since I^{131} emits highly penetrating gamma radiations, the cellular concentrations of I^{131} could be determined directly without fractionation or isolation. *Apparent metabolic activity of iodoorotic acid.* Increasing amount of iodoorotic acid were added to 2 series of assay tubes (final volume: 10 ml); of these, each tube of one series was supplemented with 50 μg of orotic acid. The tubes were autoclaved at 120°C for 15 minutes, inoculated with *L. bulgaricus* 09, and incubated for 72 hours at 37°C. Growth was measured turbidimetrically in a Klett-Summerson photoelectric colorimeter using filter No. 66, or by titration with 0.1 N NaOH.

TABLE I. Effect of Iodoorotic Acid on Growth of *L. bulgaricus* 09.

Iodoorotic acid, mg	0.1 N acid/10 ml media*	
	No addition	Plus orotic acid (50 μg)
	ml	ml
1.0	16.1	15.6
.10	12.9	15.5
.01	5.9	8.6
0	3.5	7.8

* Acid-production after 72 hr growth.

The *results*, presented in Table I, indicate that iodoorotic acid not only did not antagonize the growth of *L. bulgaricus* 09 in the presence of orotic acid, but, in the absence of the latter, enabled maximal growth to be attained.

Activity of autoclaved versus sterile-filtered solutions of iodoorotic acid. The growth of

---

* This investigation was supported by a grant from the American Cancer Society, as recommended by the Committee on Growth, National Research Council, and in part by a grant from the National Institutes of Health, U. S. Public Health Service.

† The present address of the author is Department of Pharmacology, Yale University, School of Medicine, New Haven 11, Conn.

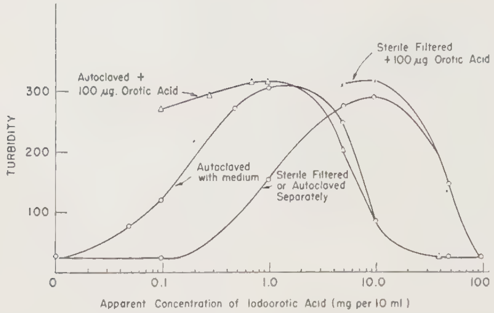


FIG. 1. Comparison of growth (45 hr growth at 37°) of *L. bulgaricus* 09 in media supplemented with iodoorotic acid which was autoclaved in the medium, or in an aqueous solution, or sterile-filtered and added aseptically.

L. bulgaricus 09 was observed: 1) when, as in the first experiment, increasing quantities of iodoorotic acid were autoclaved with the media, 2) when an aqueous neutralized solution of iodoorotic acid was autoclaved for 15 minutes at 120°C and then added aseptically to the previously autoclaved basal medium, and 3) when a neutralized aqueous solution of the iodinated pyrimidine was sterile-filtered and varying amounts were added aseptically to the autoclaved basal medium.

The 3 series of tubes were inoculated with *L. bulgaricus* 09 and incubated for 45 hours at 37°C. The results which were obtained are shown in Fig. 1.

The growth-promoting effect of 0.32 µmole of orotic acid was equalled by 0.55 µmole of iodoorotic acid which had been autoclaved with the medium, while of that which had been sterile-filtered or autoclaved as an aqueous solution, 3.58 µmoles were required. At higher concentrations the growth-promoting activity of iodoorotic acid disappeared and was replaced by a complete inhibition of growth; this effect was seen regardless of the manner of pre-treatment of the iodinated pyrimidine.

When iodoorotic acid was autoclaved in the medium in a concentration of 100 mg/10 ml of media, the solution turned a deep brown and a white precipitate formed upon cooling. No precipitate or darkening of the media was observed at this concentration of iodoorotic acid when the compound was sterile-filtered or autoclaved in an aqueous solution prior to its addition to the basal medium. The white precipitate was removed from the media by filtration, washed with 50% alcohol, then with 95% alcohol, and finally with ether, recrystallized from 50% ethyl alcohol. The spectral relationship of the compound to iodoorotic acid and orotic acid, shown in Fig. 2, suggests its identity with orotic acid. The maximum and minimum values in 0.01 N NaOH were 285 and 247 mµ, respectively (orotic acid 285 and 247 mµ), and in aqueous solution 277 and 242 mµ, respectively (orotic acid 277 and 240 mµ). As shown in Fig. 3, the growth-promoting activity of the compound for *L. bulgaricus* 09 was essentially identical with that of orotic acid.

Growth of L. bulgaricus 09 on iodoorotic acid-I^{131}. *L. bulgaricus* 09 was used to inoculate 2 one-liter flasks each of which contained 500 ml of media. The first was supplemented with sterile-filtered iodoorotic acid (165 mg/liter; 2 x 10^7 c.p.m./liter) and the second with orotic acid (20 mg/liter) plus radioactive potassium iodide-I^{131} (14 x 10^7 c.p.m./liter). The cells were harvested after 48 hours and washed 2 times with 50 volumes of 0.9% NaCl. The radioactivity contained within the cells was determined in the manner previously described. The washed cells which were grown on iodoorotic acid-I^{131} contained a total of 742 c.p.m., while those grown on KI^{131} and orotic acid had 972 c.p.m. That the activity contained in the cells grown on radioactive iodoorotic acid, as well as that observed with the cells grown on KI^{131}, could

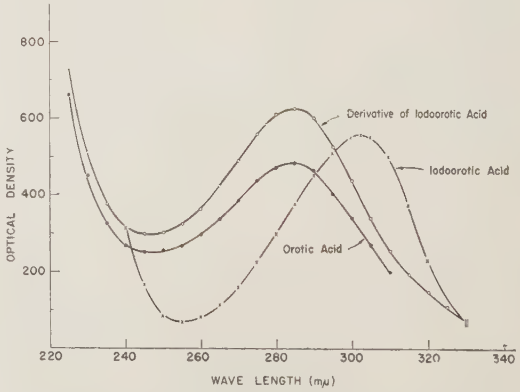


FIG. 2. Alkaline spectra of orotic acid, of iodoorotic acid, and of product formed during autoclaving of iodoorotic acid in medium.

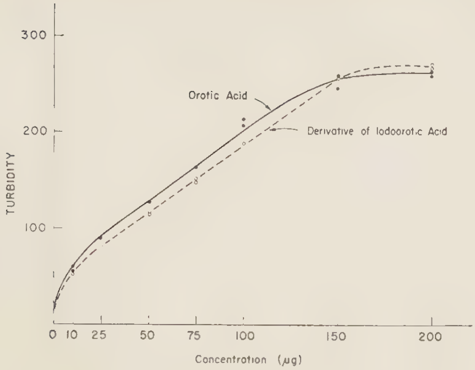


FIG. 3. Comparison of growth of *L. bulgaricus* 09 in a medium supplemented with orotic acid or a compound formed during autoclaving of iodoorotic acid in the medium.

be accounted for by the presence of iodide ion, was indicated also by the fact that more extensive washing of both types of cells removed all radioactivity.

Effect of iodide on growth of L. bulgaricus 09. Potassium iodide was added in increasing quantities to assay tubes, each of which contained 50 μg of orotic acid. After inoculation with *L. bulgaricus* 09, growth was permitted for 48 hours at 37°C. The inhibition of growth as measured turbidimetrically, which began at 4 mg/10 ml, is shown in Table II. This inhibition was not reversed by orotic acid even in concentrations of 1000 μg/10 ml media.

Metabolic activity of iodouridine and iodoorotic acid for L. casei and S. faecalis. Neither iodoorotic acid nor iodouridine, in concentrations of from 0.01 to 1.0 mg/10 ml of media, was able to support the growth of *L. casei* or *S. faecalis* in the usual basal media devoid of pteroylglutamic acid, thymine or thymidine; furthermore, the iodinated compounds were not inhibitory when growth was supported by any of these growth factors.

TABLE II. Effect of Potassium Iodide on Growth of *L. bulgaricus* 09.*

Amt of KI (in 10 ml), mg	Turbidity
40.	23 21
10.	77 84
4.0	116 122
1.0	153 156
.40	153 156
.10	153 155
0	153 157

* Medium supplemented with 50 μg orotic acid/10 ml. Turbidity, in Klett units, after 48 hr growth at 37°.

Discussion. The results indicate that iodoorotic acid is neither a growth factor nor an antagonist of the growth factor activity of orotic acid for *L. bulgaricus* 09. The pronounced growth-promoting effect of iodoorotic acid autoclaved in the microbial assay medium may be explained by a dehalogenation of iodoorotic acid with the subsequent formation of orotic acid.

When the amount of iodoorotic acid autoclaved in the medium was sufficient to yield a concentration of orotic acid greater than the solubility of that compound at room temperature, there precipitated from the medium upon cooling a substance with the properties of orotic acid. This precipitation is consistent with the fact that at pH 5.7 orotic acid is only about one-sixth as soluble as is the halogenated compound. Further, the ultraviolet absorption spectrum of the compound formed from iodoorotic acid was not significantly different from that of orotic acid. That the apparent growth-promoting activity of iodoorotic acid for *L. bulgaricus* 09 was not due to the utilization of the halogenated pyrimidine *per se* was shown by studies with iodoorotic acid-I^{131} in which the radioactive iodine was shown to behave in the same manner as KI^{131} and to be washed from the bacterial cells in a manner indistinguishable from the iodide ion. The inhibition observed with high concentrations of iodoorotic acid may have resulted from the increased iodide concentration formed by the dehalogenation of the compound. Since orotic acid is not capable of reversing the inhibition due to potassium iodide, the antagonism is considered to be of the non-competitive type. When autoclaved in the medium approximately 60% of the iodoorotic acid was converted to orotic acid, whereas an apparent conversion of only about 8% occurred when the aqueous solution of the iodinated pyrimidine was added to the medium following sterile-filtration or autoclaving. Although heating iodoorotic acid in the medium greatly accelerated the dehalogenation of this compound, the mechanism of the dehalogenation has not been investigated further.

Summary. 1. 5-Iodoorotic acid and 5-iodouridine *per se* are not utilizable for the growth of *S. faecalis* or *L. casei*, while in media supplemented with thymine, thymidine or pteroylglutamic acid, the growth of these organisms is not inhibited by iodoorotic acid or iodouridine. 2. Although 5-iodoorotic acid appeared to support the maximal growth of *L. bulgaricus* 09, evidence was presented that this was attributable to the release of orotic acid from the iodinated derivative. 3. High concentrations of 5-iodoorotic acid inhibited the growth of *L. bulgaricus* 09, but this could not be reversed by orotic acid. This inhibition was attributed to the formation of iodide ion, since potassium iodide in similar concentrations produced a comparable inhibitory effect on the microorganism.

The author wishes to express his appreciation to Dr. Arnold D. Welch for his interest in this work and for advice concerning the preparation of the manuscript.

---

1. Prusoff, W. H., Holmes, W. L., and Welch, A. D., *Cancer Research*, 1953, v13, 221.
2. Hitchings, G. H., Falco, E. A., and Sherwood, M. B., *Science*, 1945, v102, 251.
3. Von Weygand, F., Wacker, A., and Grisebach, H., *Z. für Naturforsh.*, 1951, v6b, 177.
4. Fukuhara, T. K., and Visser, D. W., *J. Biol. Chem.*, 1951, v190, 95.
5. Von Weygand, F., Wacker, A., Dellweg, H., *Z. für Naturforsh.*, 1952, v7b, 19.
6. Prusoff, W. H., PROC. SOC. EXP. BIOL. AND MED., 1954, v85, 564.
7. MacIntyre, W. J., PROC. SOC. EXP. BIOL. AND MED., 1950, v75, 561.

Received March 15, 1954. P.S.E.B.M., 1954, v85.

Enzymatic Hydrolysis of Acyl Naphthylamines.* (20954)

G. GOMORI.

From the Department of Medicine, University of Chicago.

The literature of enzymes hydrolyzing aryl-substituted acylamides such as acetanilid and phenacetin is very scanty(1-4). In the course of attempts to develop a more accurate quantitation of these enzymes, N-acetyl naphthylamines were tried as substrates because of the high color intensity of dyes obtainable from naphthylamines by azo-coupling. It was soon found that extracts of many organs will hydrolyze acetyl naphthylamines at a low rate. Taking a lead from Birnbaum and coworkers (5), and from Rao and associates(6) who found that chloroacetyl amino acids are hydrolyzed much faster than the corresponding acetyl and glycyl derivatives, chloracetyl naphthylamines were tried next as substrates. They were found to be hydrolyzed by all tissues examined 30 to 100 times faster than the corresponding acetyl compounds. The activity of some tissues was high enough to permit its localization by a histochemical method.

Experimental. Chloroacetyl naphthylamines were prepared by acylating α- and β-naphthylamine with chloroacetyl chloride in an acetone-pyridine medium. The compounds were recrystallized twice from 60% methanol. The corrected melting points are: chloroacetyl α-naphthylamine, 161°C; chloroacetyl β-naphthylamine, 113°C. 0.03 M stock solutions in acetone were prepared of both substances. For use, one ml of the stock solution was blown into 100 ml of 0.04 M buffer. Both the stock solutions and the buffered substrate mixture were found to be stable at room temperature for several weeks. The enzymes used were 1:10 homogenates of fresh human tissues in saline, cleared by the freezing-thawing method(7), and human serum. One ml of enzyme dilution was incubated with 5 ml of buffered substrate for

---

* This work has been done under grants from The Douglas Smith Foundation for Medical Research of The University of Chicago and from The Pathology Study Section of the U. S. Public Health Service.

TABLE I. Hydrolysis of Chloroacetyl α-naphthylamine by Extract of Human Pancreas. Dependence on pH. Acetate (pH 4.25 to 5.45) or phosphate buffers. 4 mg of tissues/tube. Incubation: 1 hr. Values in percentages of hydrolysis at pH 6.2.

pH	4.25	4.7	5.0	5.45	5.8	6.0	6.2	6.4	6.6	7.15	8.0
% of hydrolysis	19	20	25	41	88	98	100	92	84	57	4

various lengths of time. At the end of incubation the tubes were placed in ice water, and one ml of enzyme was added to the control tubes, incubated without enzyme. One ml of a M acetate buffer of pH 4.2 to 4.5 containing 10% of Tween 20 or 40 was added to the tubes, followed by 0.5 ml of a 0.25 to 0.5% fresh solution of Diazo Red B Salt. The tubes were inverted twice and allowed to stand. They were read between 5 and 15 minutes at 550 mμ against their controls. A curve was standardized with dilutions of naphthylamines containing 2 to 25 μg ($\pm$0.02 to 0.2 μ M) of naphthylamine in 5 ml. The reason for using Tween in the solution is its ability to keep the azo dye, utterly insoluble in aqueous media, in a perfectly clear colloidal solution, suitable for direct colorimetry. This principle has been recommended also for other azo dye methods(7).

Results. 1) *pH optimum.* The dependence of the rate of hydrolysis of chloroacetyl α-naphthylamine by human pancreas is shown in Table I. All subsequent experiments were run at pH 6.0 to 6.2.

2) *Rates of hydrolysis of acyl naphthylamines.* Table II shows the rates of hydrolysis of acetyl and chloroacetyl naphthylamines in terms of μg of naphthylamine liberated per hour and per mg of tissue. The much higher rates of hydrolysis of the chloroacetyl compounds is clearly shown; also, the relative preference of the pancreatic enzyme for the β-isomers.

TABLE II. Hydrolysis of Acyl Naphthylamines by Human Tissues. μg of naphthylamine liberated /hr/g of tissue.

	Acetyl α-naphthylamine	Acetyl β-naphthylamine	Cl-acetyl α-naphthylamine	Cl-acetyl β-naphthylamine
Kidney	20	0	640	160
Liver	130	186	4600	1650
Pancreas	13	27	1500	1800

3) *Proportionality between the amount of enzyme and the extent of hydrolysis.* Between 0.01 and 0.15 μM of substrate hydrolyzed in 2 hours, the extent of hydrolysis was proportional to the amount of enzyme used.

4) *Course of hydrolysis in time.* As shown in Table III, during the first few hours of hydrolysis there is a slight gradual inactivation of the enzyme.

TABLE III. Course of Hydrolysis in Time. Human pancreas, 2 mg/tube. μg naphthylamine liberated.

Hr	¼	½	1	2	3	4	5	8	12	24
μg	1.0	2.0	3.5	6.0	8.7	11.3	14	21.2	32.2	63.5

5) *Inhibition effects.* As shown in Table IV, a number of metal ions and taurocholate have a definite inhibitory effect on the enzyme. So far, no activating substances have been found.

6) *Hydrolysis of chloroacetyl α-naphthylamine by human serum.* To date, 30 unselected human sera have been tested for their activity. As a rule, one ml of a 50% dilution of serum was used; length of incubation was 2 hours. Table V shows the activity range of human sera.

7) *The effect of other enzymes.* None of the substrates mentioned were hydrolyzed measurably by the following enzymes: one mg crystalline pepsin (at pH 6.2 and 2.8); one mg crystalline chymotrypsin; 5 mg of crude trypsin or papain; 8 samples of human

TABLE IV. Inhibition Effects. Human liver; 4 mg/tube. 1 hr values.

Addition	None	Ca, 0.01M	Co, 0.01M	Mg, 0.01M	Mn, 0.002M	Taurocholate, 0.004M
μg naphthylamine liberated	18	14.6	10.6	13.2	8.6	5.6

TABLE V. Hydrolysis of Chloroacetyl α-naphthylamine by Human Serum. Range of activity.

μg of naphthylamine liberated /hr/ml serum	0-1.9	2-3.9	4-5.9	6-7.9	8-11
No. of sera	4	13	6	3	4

gastric juice.

8) *The histochemical localization of activity.* When paraffin sections of acetone-fixed tissues were incubated with the chloroacetyl α-naphthylamine substrate mixture in the presence of about 20 mg of Diazo Garnet GBC Salt per Coplin jar (a method analogous to those used for the demonstration of esterases (8), moderately intense but definite localizations were obtained within 30 minutes to 2 hours in some tissues. Owing to the small number of tissues examined, no general statement on the distribution of enzymatic activity can be made at this time. So far, activity could be demonstrated at the following sites: liver and intestinal mucosa of the guinea pig; intestinal mucosa of the rat; bronchial mucosa and convoluted renal tubules of the cat (see Fig. 1 to 3). Human tissues, quite active in the test tube, were invariably inactive in paraffin sections.

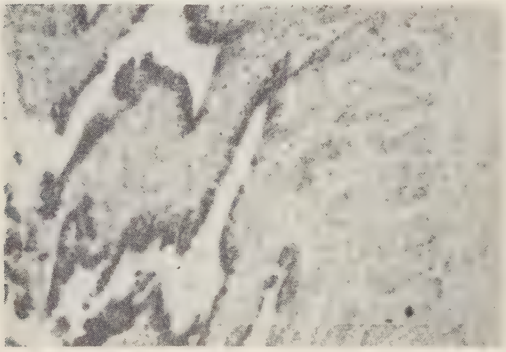


FIG. 1. Intestine of guinea pig. Activity at surface of villi.

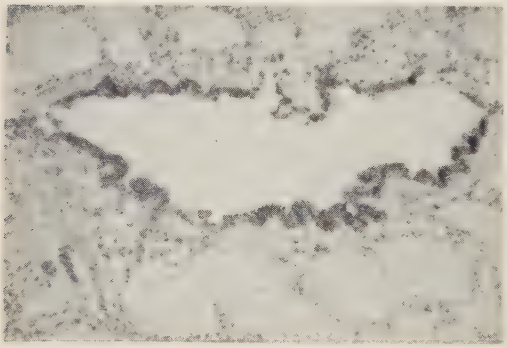


FIG. 2. Lung of cat. Activity in bronchial epithelium.

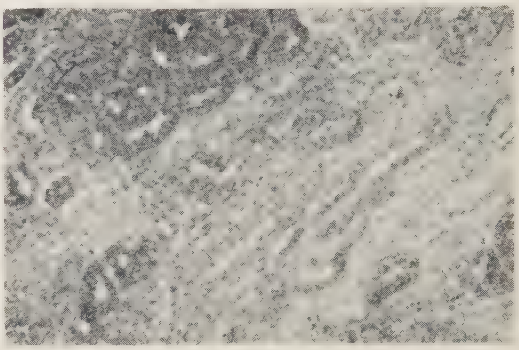


FIG. 3. Kidney of cat. Activity in convoluted tubules.

Conclusions. An enzyme, hydrolyzing acyl naphthylamines, can be demonstrated in a number of tissues and in serum. Chloroacetyl naphthylamines are hydrolyzed at a much higher rate than the corresponding acetyl compounds. It is possible to localize the enzyme histochemically in some tissues.

1. Michel, H. O., Bernheim, F., and Bernheim, M. L. C., *J. Pharm. and Exp. Ther.*, 1937, v61, 321.

2. Greenberg, L. A., and Lester D., *ibid.*, 1946, v88, 87.

3. Smith, J. N., and Williams, R. T., *Biochem. J.*, 1948, v42, 538.

4. Bray, H. G., and Thorpe, W. V., *ibid.*, 1948, v43, 211.

5. Birnbaum, S. M., Levintow, L., Kingsley, R. B., and Greenstein, J. P., *J. Biol. Chem.*, 1952, v194, 455.

6. Rao, K. R., Birnbaum, S. M., Kingsley, R. B., and Greenstein, J. P., *ibid.*, 1952, v198, 507.

7. Gomori, G., *J. Lab. and Clin. Med.*, 1953, v42, 445.

8. ———, *Microscopic histochemistry. Principles and practice.* University of Chicago Press, Chicago, 1952.

Received March 17, 1954. P.S.E.B.M., 1954, v85.

Effect of Sedormid on the Developing Chick Embryo.* (20955)

Arthur W. Frisch, Ellen L. Talman, Robert A. Aldrich, Richard A. Nevé,
and James D. Case.

From the Departments of Bacteriology, Biochemistry, and Pediatrics, University of Oregon Medical School, Portland.

The ability of Sedormid (allylisopropylacetyl carbamide) to produce acute porphyria in man was suggested by the findings of Duesberg(1). A similar disorder has recently been induced in rabbits(2) and rats(3) by the oral administration of the same drug. The purpose of the present communication is to report the appearance of abnormal amounts of porphyrin-like substances in embryonated eggs receiving Sedormid in the yolk sac or in the allantoic cavity.

Materials and methods. White Leghorn eggs were used in all experiments. Sterile suspensions of Sedormid were prepared in final concentrations of 20 to 40 mg/ml as follows: 1) Weighed samples of Sedormid were suspended in isotonic (0.3 molar) glycerol containing 0.1% Tween 80 in a mortar or a Waring blendor. Suitable volumes of the suspension were transferred to vaccine vials and sterilized by heating in a boiling water bath for one hour on each of 3 successive days. The vials were shaken frequently during heating and upon removal were placed in a shaking machine until cool. 2) Weighed samples of Sedormid were transferred to cotton stoppered flasks and sterilized in a hot air oven at 160°C for one hour. After sterilization of the glycerol-Tween 80 solution by Seitz filtration, the 2 reagents were homogenized in a sterile mortar and transferred to sterile vaccine vials. Appropriate volumes of such sterile suspensions were injected into the yolk or allantoic sacs of 8-day embryonated eggs in the manner usually employed for viral inoculations. The maximum volume given was 0.75 ml. Control eggs received 0.75 ml of glycerol-Tween 80 at the same time. Eggs were incubated at 37°C and candled daily for viability. At convenient intervals after inoculation several eggs from each group were chilled at 0°C for one hour to prevent bleeding, the shell over the air sac cut away and the allantoic fluids aspirated with a syringe and needle. Each fluid was harvested separately, centrifuged and a measured aliquot was included as part of a final pool for any given lot of eggs. Pools were composed of allantoic fluids from 3 to 6 living embryos. After addition of 3 ml of 10% trichloroacetic acid to 5 ml of each pool and removal of the precipitate by centrifugation, the supernate was adjusted to pH 6-7 with NaOH and brought to a volume of 10 ml. A 5-ml aliquot of this solution was used for the determination of coproporphyrin by the method of Schwartz *et al.*(4). Uroporphyrin was estimated in the aqueous phase from the coproporphyrin determination as outlined by Case *et al.*(3). Since allantoic fluid contains little protein if trauma to blood vessels is avoided, an estimate of the total amount of fluorescent material present was obtained by treating 2.5 ml of the pool with 5 ml of 7.5 N HCl, removing the precipitate by centrifugation, diluting the supernatant to 25 ml with distilled water, and reading the resulting solution in the fluorimeter against a coproporphyrin standard.

Results. The data which were obtained at varying intervals following the inoculation of 15 mg of Sedormid per embryo via the allantoic cavity and via the yolk sac are presented in Table I. The results in the first 2 columns are expressed in terms of a coproporphyrin standard either as μg of uro or coproporphyrin per 100 ml of pooled allantoic fluid. The third column indicates the sum of these 2 determinations and is compared with the values for total porphyrin as measured by the technic given. Elevated porphyrin levels were first noted 64 hours after the introduction of the drug into the embryos and these were maintained for the duration of the experiment

---

* Supported by the Office of Naval Research, Oregon Heart Association and the Helen Hay Whitney Foundation.

TABLE I. Porphyrin Levels in Allantoic Fluids of Chick Embryos Given 15 mg Sedormid: Yolk Versus Allantoic Cavity as Route of Inoculation.

Incubation, hr	Control				Inoculation route yolk sac				Allantoic cavity			
	Uro*	Copro	Sum	Total	Uro*	Copro	Sum	Total	Uro*	Copro	Sum	Total
40	0	1	1	—	0	3	3	10	0	1	1	—
64	0	1	1	3	7	15	22	23	13	27	40	53
88	0	3	3	4	29	77	106	—	25	68	93	—
112	0	2	2	2	26	54	80	96	36	93	129	129
160	1	5	6	10	50	61	111	138	72	136	208	183
184	9	2	11	14	420	270	690	520	220	180	400	454
234	14	2	16	21	295	95	390	308	375	353	728	630

Values expressed as μg of coproporphyrin/100 ml allantoic fluid.
* Unheated specimens.

170 hours later. The values for total fluorescence showed a similar rise.

In the second experiment varying doses of Sedormid were given into the allantoic cavity of 8-day embryos and the allantoic fluids were harvested at 3 intervals as shown in Table II. Assays for total porphyrin were obtained at 40, 112, and 160 hours; partition into uro-type and coproporphyrin was done on the 112-hour pool only. A distinct elevation in total fluorescence was noted at the end of 40 hours and with as little as 5 mg Sedormid per egg. The 10-mg dose was as effective in eliciting a maximal response as larger amounts of drug and was less toxic to the embryos. With time there was a gradual and progressive accumulation of porphyrin-like substances in the allantoic cavity. A portion, but not all, of the increase may be accounted for by the reduction in the volume of allantoic fluid which normally occurs between the 14th and 16th day of embryonic development.

The outstanding effects of Sedormid on the developing chick were a decrease in size of the embryo and the appearance of edema. Three groups of embryos are shown in Fig. 1, 160 hours after the inoculation of 15 ml of Sedormid. The greatest stunting was observed in the eggs injected into the yolk sac (Y) and the 3 illustrated weighed a total of 19 g. Those receiving the drug into the allantoic cavity (A) were slightly larger and weighed 22 g. The control embryos (C) totaled 30 g. In addition doses of 20 and 30 mg of Sedormid induced a marked edema which was generalized in character. At this level of drug significant degrees of hemorrhage were noted over the surface of the embryos and occasionally in the brain itself. The organs were not grossly altered but the livers were friable and some were dark green in color. Those embryos which manifested edema were extremely friable indicating that the changes could have been terminal even though the chicks were alive at the time the allantoic fluids were harvested. Examination of the chicks under the ultraviolet light revealed gross, red fluorescence in the kidneys, sternum, and long bones. Studies of tissue components will be reported separately.

TABLE II. Porphyrin Levels in Allantoic Fluids of Embryos Given Varying Doses of Sedormid.

Dosage (mg)	40 hr pool total	112 hr pool				160 hr pool total
		Uro	Copro	Sum	Total	
30	23	64	80	144	147	—
20	43	100	80	180	168	435
15	32	60	66	126	108	263
10	40	100	97	197	138	428
5	15	18	26	44	27	345
1	5	0	1	1	3	30
0	3	—	—	—	—	10

μg/100 ml allantoic fluid.

Discussion. Protoporphyrin has been identified in the eggshell(5,6), and eggshell membranes(7), in the yolk(8), and in the albumin(9) of the developing hen's egg. Schønheyder(10) has crystallized coproporphyrin I isolated from chick embryos. No studies dealing with porphyrins in allantoic fluid have been reported.

The general pattern of the response of chick embryos to Sedormid treatment corresponds to that of rabbits(2) and rats(3). However, since none of the porphyrins have been isolated for spectrophotometric study and identification of their isomeric configurations, the

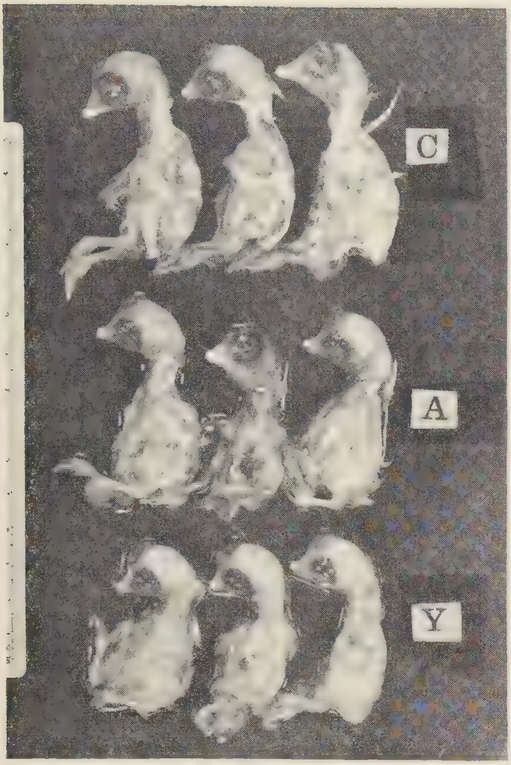


FIG. 1. Chick embryos given Sedormid. C = controls; A = 15 mg intra-allantoic; Y = 15 mg via yolk sac.

production of an experimental porphyria in chick embryos has not yet been established. The data thus far obtained establish only that Sedormid produces a metabolic disturbance resulting in the appearance of considerable quantities of fluorescent material in the allantoic fluids of treated eggs within a very short time. The fluorescent materials observed in both control and Sedormid-treated eggs will probably prove to be porphyrins but further interpretations based upon these data are not justified until the porphyrins present have been isolated and identified and their isomer types established. If the porphyrins from Sedormid-treated eggs are of the proper isomer type (Type III) to allow this disorder to be labeled acute porphyria, the chick embryo offers several advantages for studies of porphyrin metabolism, one of the most important of these being its status as a convenient, inexpensive, bacteria-free system.

Summary. The inoculation of Sedormid into the yolk sacs or allantoic cavities of 8-day-old embryonated eggs results in the prompt appearance in their allantoic fluids of materials exhibiting red fluorescence in ultraviolet light. These fluorescent materials appear to accumulate progressively with time. The results of quantitative estimations suggest that coproporphyrin and uroporphyrin are responsible for this fluorescence. Treated embryos are smaller than the controls and exhibit red fluorescence of the kidneys, sternum, and long bones under ultraviolet light. Further interpretation of these data must await isolation and characterization of the fluorescent substances.

The technical assistance of Mr. Merle Marriott is gratefully acknowledged.

---

1. Duesberg, R., *Münchener Med. Wchschr.,* 1932, v79, 1821.

2. Schmid, R., and Schwartz, S., PROC. SOC. EXP. BIOL. AND MED., 1952, v81, 689.

3. Case, J. D., Aldrich, R. A., and Nevé, R. A., *ibid.,* 1953, v83, 566.

4. Schwartz, S., Zieve, L., and Watson, C. J., *J. Lab. Clin. Med.,* 1951, v37, 843.

5. Fischer, H., and Lindner, F. Z., *Physiol. Chem.,* 1925, v142, 141.

6. Voelker, O., *J. Ornithology,* 1940, v88, 604.

7. Klose, A. A., and Almquist, H. J., *Poultry Sci.,* 1937, v16, 173.

8. Gouzon, B., *Compt. rend. Soc. Biol.,* 1934, v116, 925.

9. Van den Bergh, A. A., and Grotepass, W., *Compt. rend. Soc. Biol.,* 1936, v121, 1253.

10. Schønheyder, F., *J. Biol. Chem.,* 1938, v123, 491.

Received December 29, 1953. P.S.E.B.M., 1954, v85.

Fermentable Chromogen in Ampuled Inulin and Its Renal Clearance. (20956)

MICHAEL LADD AND JOHN GAGNON. (Introduced by Robert W. Clarke.)

From the Department of Surgical Physiology, Army Medical Service Graduate School, Washington, D. C.

The resorcinol method of Schreiner(1) for the determination of inulin is simple because it does not entail yeasting. In the past when ampuled inulin was used, this omission seemed justified since the inulin contained only 2% fermentable material(2). However, in recent dog experiments high creatinine-inulin clearance ratios were noted whenever ampuled inulin was measured by this method without yeasting. We have tested 5 lots of inulin which were available and found up to 50% fermentable material to be present. The significance of this finding in renal clearance studies is apparent in the data given below.

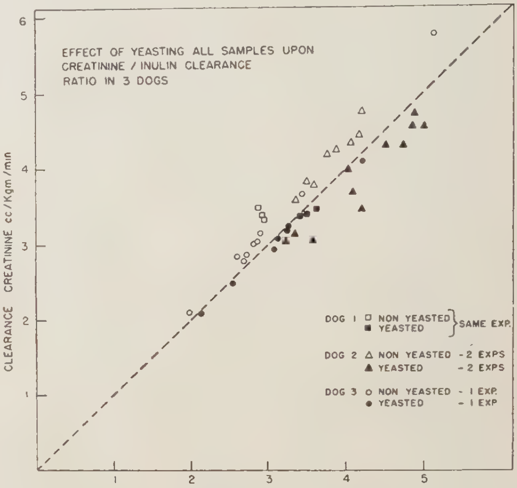


FIG. 1.

Methods. Three or more clearance periods were obtained in each experiment, anesthetized dogs (nembutal) being used. Approximately level plasma concentrations of test substances, inulin 30 mg % and creatinine 20 mg % were maintained by a constant infusion pump. Concomitantly, a saline infusion insured a steady urine flow approximating 3% of the estimated filtration rate. Creatinine was determined in trichloracetic acid filtrates of both plasma and urine(3) and inulin in yeasted and non-yeasted cadmium sulfate filtrates of both plasma and urine. Urine samples were diluted to an expected U/P ratio of one, and in the final calculations, predetermined urine and plasma blanks were subtracted from experimental values. Water recoveries were performed as follows: Ampules of inulin were first warmed at temperatures below 100°C. Buffered inulin (USS inulin*) invariably went into solution quickly. Ampules containing no buffer (WW inulin), usually required about 10 minutes of heating before all crystals went into solution. After cooling, stock solutions of 20 mg % were prepared from each ampule. Further dilutions were subjected to acid hydrolysis and colored by resorcinol(1) both without yeasting and after yeasting. Yeasting recoveries were carried out by adding 2 ml of 20 mg % inulin to 8 ml of yeast suspension having a measured concentration of yeast cells. The mixture was allowed to stand with occasional shaking for 15 minutes at room temperature after which it was centrifuged for 10 minutes at 2000 g. The yeast blank from fresh Fleischman's yeast, washed 10 to 13 times before use, rarely contained significant amounts of reducing substance.

Results. Data from 7 experiments on dogs shown in Fig. 1 show that treating both plasma and urine samples with 40% yeast suspension raises the inulin clearance and the inulin/creatinine clearance ratio.

The variations among the 2 sets of data in Fig. 1 (open and closed symbols) are attributed to individual differences between animals and to differences in the amount of fermentable chromogen in various lots of inulin.†

---

* Preparations are "USS" = U. S. Standard Products inulin, Woodworth, Wisc. "WW" = William Warner inulin, Warner-Chilcott Laboratories, New York City.

† Unfortunately, the lot numbers were not recorded for each experiment, but we subsequently have examined each lot that was available to us.

TABLE I. Effect of Yeasting on Renal Clearance Data in a 19.1 kg Dog.

	Collection period			
	0	1	2	3
Urine inulin, mg %				
Not yeasted	2.8	474	413	496
Yeasted	5.5	464	398	480
Plasma inulin, mg %				
Not yeasted	.96	20.8	20.0	20.0
Yeasted	.46	16.6	16.0	15.0
Fermentable chromogen, %				
Urine		2.1	3.6	3.2
Plasma		20.2	20.0	25.0
Urine flow, ml/min.	.82	2.29	2.53	2.09
Clearances, ml/min.				
Inulin, not yeasted	2.4	54.6	54.8	54.4
" , yeasted	9.8	65.8	64.7	69.0
Creatinine		64.9	64.2	66.4
C_{cr}/C_{in}				
Not yeasted		1.19	1.17	1.22
Yeasted		.99	.99	.96

Table I gives the data for 3 urine collection periods in one representative experiment. If it is assumed that the fermentable component is freely filterable, its partial failure to appear in the urine must reflect tubular reabsorption.

The importance of yeast concentration in the analysis for inulin was studied by noting the relationship between the amount of inulin lost and yeast used. This was found to vary with each of the different lots of inulin. Fig. 2 summarizes the results, but no precise quantitative significance should be given to the several curves. Because different ampules agree reasonably closely within a given lot of either brand it is evident that the contaminating material is characteristic of entire lots rather than occasional ampules.

Even the more homogeneous lots showed an increasing loss of inuloid chromogen when the proportion of cells in the yeast suspension was raised from 10 to 40%. A similar phenomenon has been observed with respect to non-fermentable reducing substances occurring in blood(4) and with xylose(5) and has been attributed to partial permeation of these substances into the intracellular water of yeast cells. The curvilinear slope for yeast absorption seen in Fig. 2 could reflect several processes including fermentation of fructose and oligosaccharides, and the action of small amounts of specific inulinase, as well as diffusion.

We have made no attempt to determine the more exact nature of the fermentable material present in commercial preparations. Cotlove (personal communication) has found 26% copper reducing activity (in fructose equivalents) in one lot of the material discussed here (USS lot No. 229A1) and on paper chromatography, fructose, disaccharides and higher oligosaccharides were present in significant amounts. Most available inulin (powdered or ampuled) apparently contains 15 to 40% alkali labile material diffusing faster than the alkali stable material which behaves like a larger molecule. Young and Raisz(6) have compared the simultaneous renal clearances of these 2 fractions in the dog and found them to be equal. Free fructose could conceivably be produced by hydrolysis of inulin within the ampule, during warm storage of ampules containing a slightly acid buffer. We have noted no relationship between fermentable chromogen content and storage time.

Summary. A comparison has been made of the fermentable chromogen content in several lots of 2 commercial brands of ampuled inulin. It is shown that as much as 50% of some currently available ampuled inulin cannot be recovered after mixing with yeast. A lower creatinine/ampuled-inulin clearance ratio has been found in dogs when samples of plasma and urine are treated with yeast prior to analysis. This indicates that when present, fermentable material is probably reab-

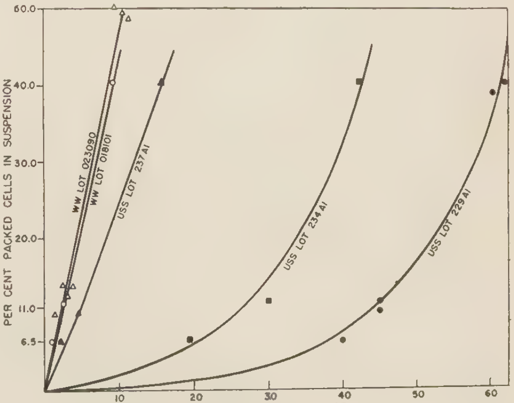


FIG. 2.

sorbed by the renal tubule. Ampuled inulin suspected to contain it should not be used for measuring filtration rate unless all samples are yeasted before analysis.

---

1. Schreiner, G. E., PROC. SOC. EXP. BIOL. AND MED., 1950, v74, 117.

2. Ladd, M., *J. Applied Physiol.*, 1951, v3, 379.

3. Kennedy, T. J., Jr., Hilton, J. G., and Berliner, R. W., *Am. J. Physiol.*, 1952, v171, 164.

4. Hawkins, J. A., and Van Slyke, D. D., *J. Biol. Chem.*, 1929, v83, 51.

5. Jolliffe, N., Shannon, J. A., and Smith, H. W., *Am. J. Physiol.*, 1932, v100, 301.

6. Young, M. K., Jr., and Raisz, L. G., PROC. SOC. EXP. BIOL. AND MED., 1952, v80, 771.

Received December 11, 1953. P.S.E.B.M., 1954, v85.

Inhibition of Serous Exudation and Tumor Cell Proliferation in Peritoneal Cavities of Mice Given Cortisone.* (20957)

HORACE GOLDIE, MATTHEW WALKER, ARNOLD M. JONES, AND DAVID E. ROSS.

From the Laboratory for Experimental Oncology and Department of Surgery, Meharry Medical College, Nashville, Tenn.

Previous tests for effects of cortisone on various mouse tumors (including Sarcoma 180) have revealed as a rule no inhibition of their growth(1-3). Moreover, several reports have stated that cortisone treatment facilitated the growth of transplanted tumor cells (4) by modifying host environment, *i.e.*, by "diminishing the awareness of the host"(5) or by inhibiting antibody formation(6). On the other hand, some authors working with different tumors and under different experimental conditions recorded complete(7) or moderate(8-10) inhibition of tumor growth. However, no investigation on this problem has thus far been carried out with mouse tumors growing in a peritoneal exudate and on the serosa. Sarcoma 180 cells can be cultured *in vivo* in this way(11,12), and it occurred to us that these experimental conditions might provide a suitable tool for measuring quantitatively the effect of cortisone both on tumor cells and on their environment. Accordingly, we have inoculated mice i.p. with requisite numbers of Sarcoma 180 cells and treated some of them with cortisone while keeping others as controls. Later, the amount and cellular composition of the exudate in the peritoneal cavities of the experimental and control mice were determined, as was also the amount of tumor implanted on the serosa. The results are reported below.

Material and methods. 1. *Tumor and animal strains.* We have previously described (12) the ability of S-180 to grow as free cells in serial transfers in the peritoneal or pleural exudate of CFW mice. 2. *Inoculation and exploratory abdominal puncture.* Doses of inoculated tumor cells were high enough (10 to 15 millions) to insure takes and to reduce individual variations. Technics of inoculation, of counting tumor cells and of measuring the amount of peritoneal exudate by means of glass capillaries have been described elsewhere(11). 3. *Treatment.* Cortisone and hydrocortisone, both alcohol free and acetate preparations[†] were used, but since they produced similar effects the results were pooled. All mice received the same total dose of cortisone (5 mg); this was started on the day of implantation and given in one or 2 daily doses either of one mg (concentrated treatment) or of 0.5 mg (fractionated treatment). Control series of treatments were given to mice with peritoneal exudates induced by the i.p. injection of normal tissue cells (ground liver, spleen, lung, and brain) in order to study the effect of cortisone on exudate devoid

---

* This investigation was supported by a grant from the National Cancer Institute, NIH, U. S. Public Health Service and by the Division of Biology and Medicine, Atomic Energy Commission.

[†] Cortisone preparations were obtained by courtesy of Dr. Elmer Alpert, Medical Research Division, Merck & Co.

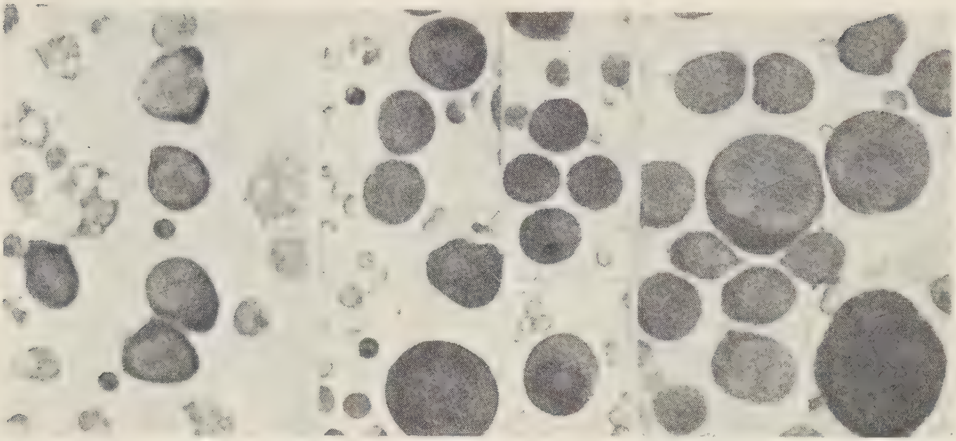


FIG. 1. S-180 cells and leukocytes in peritoneal fluid of CFW mice after treatment with cortisone or untreated. Left: untreated; center: treated with 5 × 1 mg cortisone i.p.; right: treated with 5 × 1 mg cortisone s.c. Wright's stain × 450. Note: (a) abundance of macrophages in the exudate of untreated mice; (b) "swollen" cells and abnormal mitoses in treated mice; (c) polymorphonuclears in i.p. treated, scarcity of leukocytes in s.c. treated.

of tumor cells.[‡]

Results. Implanted but untreated control mice, and those treated by the fractionated method, died consistently after 8 to 10 days. About 15 to 20% of mice treated by the concentrated method died on the 7th day. All control animals injected i.p. with normal tissue cells survived the concentrated treatment, even when 3 additional daily doses of one mg were given.

Specimens of exudate withdrawn on the third day from mice given the "concentrated" treatment showed consistently a considerable number of "swollen" tumor cells, some with abnormal mitoses in the prophase stage of division (Fig. 1). Transfer of these specimens into new hosts resulted in tumor cell growths quite similar to those resulting from peritoneal exudates of untreated mice.

Further results of the experiments are given in detail in Table I. From the findings it appears that the "concentrated" treatment, and in a lesser degree the "fractionated" treatment also, decreased the amount of the peritoneal exudate, sometimes below the level of detection by exploratory puncture. This effect was not specific for exudate induced by tumor cells since it was observed also in mice with exudate provoked by normal tissue cells. The decrease in the amount of exudate was reflected in several mice by higher concentration of its cells, *i.e.*, higher cell counts per cmm, but in most of the cortisone-treated mice these counts were lower than in controls. In third-day counts, for example, the percentage of tumor cells was higher in treated animals than in the controls, owing to a striking depression of lymphocytes (which was observed also in the control group 2 with exudate induced by normal cells). However, on the 7th day the proportions were reversed and the decrease both in total number of cells and in number of tumor cells indicated inhibition of growth of the tumor cells. Furthermore, gross implantation on the serosa was completely inhibited by the "concentrated" treatment (group 1, series 1, Table I) in a large proportion of the cases and more or less inhibited in the remainder (Fig. 2), and partial inhibition was effected by the "fractionated" treatment also, as was revealed by small size and early necrosis of the implants in this group. Our results indicate that cortisone suppresses not only defensive tissue reactions (antibody formation, leukocytosis(5,6)) but also those which are unfavorable for the host, in our experiments, the exudate formation promoting tumor cell growth and spread. Our experiments deal with a special type of growth in a fluid medium enclosed in a serous cavity,

---

[‡] Mr. Wendell Gillett, medical photographer at Vanderbilt Medical School, prepared the photographs.

TABLE I. Effects of Cortisone on Induction of Peritoneal Exudate by S-180 Cells and on Growth and Implantation of These Cells in the Peritoneum of the Mouse.

	Date of examination	Implanted but untreated (controls)	Implanted and treated with cortisone Intraperitoneally	Implanted and treated with cortisone Subcutaneously
Group 1—Mice implanted i.p. with S-180 cells				
			First series ''conc. treatment'' 5 mg in 48 hr	
Amt peritoneal exudate	A	100% +++	8% ++, 58% +, 34% 0	54% +, 46% 0
	B	100% +++	32% ++, 56% +, 12% 0	18% ++, 64% +, 18% 0
Total cell No. (thousands/mm³)	A	86 (15– 55)	152 (55–199)	210 (85–345)
	B	75 (34–106)	85 (53–119)	64 (24– 84)
% tumor cells	A	58 (35– 64)	72 (45– 83)	78 (51– 88)
% lymphocytes		27 (21– 35)	3 (0– 12)	9 (0– 14)
% monocytes and macrophages		15 (8– 39)	8 (2– 14)	12 (4– 25)
% polymorphs		6 (3– 12)	21 (11– 33)	11 (4– 22)
% tumor cells	B	64 (49– 78)	35 (28– 49)	47 (22– 61)
% lymphocytes		26 (15– 52)	32 (15– 41)	28 (9– 37)
% monocytes and macrophages		20 (8– 33)	11 (8– 19)	19 (13– 27)
% polymorphs		6 (2– 14)	28 (8– 41)	10 (4– 19)
Amt peritoneal implant growth	B	76% +++, 24% ++	68% +, 32% 0	46% +, 54% 0
			Second series ''fractionated treatment'' 5 mg cortisone in 6 days	
Amt peritoneal exudate	B	100% +++	36% +++, 64% ++	44% +++, 56% ++
Total cell No. (thousands/mm³)	B	123 (75–276)	72 (34–178)	66 (24–184)
% tumor cells		41 (27– 56)	46 (18– 63)	62 (35– 70)
% lymphocytes		24 (14– 31)	12 (5– 20)	17 (13– 24)
% monocytes and macrophages		34 (10– 49)	16 (8– 22)	12 (8– 21)
% polymorphs		12 (7– 24)	34 (5– 57)	20 (7– 31)
Amt peritoneal implant growth	B	76% +++, 24% ++	22% ++, 78% +	28% ++, 72% +
Group 2—Controls: exudate induced by inj. of normal tissue cells; treated with cortisone				
Amt peritoneal exudate	A	22% +++, 78% ++	72% +, 28% 0	32% +, 68% 0
Total cell No. (thousands/mm³)	A	157 (107–191)	213 (188–272)	243 (185–292)
% lymphocytes		44 (15– 62)	28 (9– 47)	32 (17– 45)
% monocytes and macrophages		42 (18– 78)	36 (14– 60)	44 (10– 56)
% polymorphs		22 (14– 30)	53 (23– 75)	32 (11– 55)

Group 3—Controls: not implanted, untreated

Amt of peritoneal exudate	Total cell No. (thousands/mm³)	% of lymphocyte	% of monocytes and macrophages	% of polymorphs
.8% ++, 92% +	93 (63–160)	72 (61–81)	32 (12–51)	.6 (0–1.5)

Fifty mice in each experiment of first 2 series (group 1), 30 in group 2 and 20 in group 3. For total numbers and differential counts averages and extremes are indicated. Amount of exudate estimated* as proportionate to volume of fluid obtained by insertion of glass capillary in peritoneal wall: +++ $= 0.5$ mm³ or more, ++ $= <0.5$ but >0.1 mm³, + $= <0.1$ mm³, 0 = no fluid. Condition of peritoneal implants graded as follows: +++ = lumps of healthy tumor tissue, >0.5 cm in each diameter; ++ = <that size or partly necrotic tumors; + = mostly necrotic nodules; 0 = no implant. Exudate withdrawn on 3rd (A) or 7th (B) day after inoculation. All autopsies performed on 7th or 8th days (B).

---

* The reliability of this estimate was shown by comparison with the amount of exudate measured at autopsy.

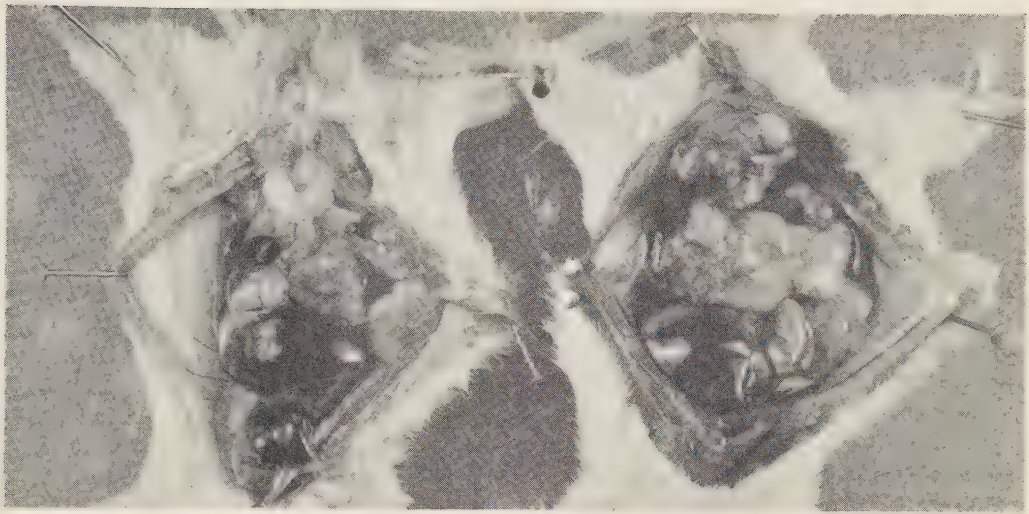


FIG. 2. Absence of large peritoneal S-180 implants in CFW mice treated with cortisone: Mouse 1 (left) = untreated, 2 (right) = i.p. treated with 5 × 1 mg cortisone.

but similar results may be demonstrated conceivably in other conditions of tumor growth.

Summary and comment. The results as a whole indicate that the treatment with cortisone impaired the growth of intraperitoneally implanted Sarcoma 180 cells, as was shown by morphological abnormalities in the tumor cells of treated animals, by a decrease in the number of tumor cells present in the peritoneal exudate, and by the lack or scarcity of serosal implants. This effect was not due to direct contact between cortisone and tumor cells, since i.p. treatment was not more efficient than s.c. injections. Furthermore, tumor cells of cortisone-treated mice grew freely when transferred into new hosts. Hence, it seems reasonable to suppose that the cortisone influenced the tumor cells only indirectly by changing their environment unfavorably. The essential change appeared to consist in a suppression of the serous exudate which is induced by the presence of tumor cells in the peritoneal cavity and constitutes a necessary condition for their growth and implantation. The scarcity or lack of serous fluid in spite of abundant growth of free tumor cells in the peritoneal cavity may be attributed to the well known suppressing action of cortisone on capillary permeability(13,14).

---

1. Sugiura, K., Stock, C., Dobriner, K., and Rhoads, C., *Cancer Res.,* 1950, v10, 244.

2. Monsen, H., *ibid.,* 1952, v12, 284.

3. Gottschalk, R. G., and Grollman, A., *ibid.,* 1952, v12, 651.

4. Toolan, H. W., *ibid.,* 1953, v13, 389.

5. Howes, E. L., *Yale J. Biol. and Med.,* 1951, v23, 461.

6. Green, H. N., and Whiteley, H. J., *Brit. Med. J.,* 1952, v2, 538.

7. Heilman, F. R., and Kendal, E. C., *Endocrinology,* 1944, v34, 416.

8. Burchenal, H. H., Stock, C. C., and Rhoads, C. P., *Cancer Res.,* 1950, v10, 209.

9. Bloom, F., PROC. SOC. EXP. BIOL. AND MED., 1952, v79, 651.

10. Stoerk, H. C., *ibid.,* 1950, v74, 798.

11. Goldie, H., and Felix, M., *Cancer Res.,* 1951, v11, 73.

12. Goldie, H., Jeffries, B. R., Maxwell, M. C., and Hahn, P. F., *ibid.,* 1952, v12, 422.

13. Ebert, R. H., and Wissler, R. W., *J. Lab. Clin. Med.,* 1951, v38, 497.

14. Menkin, V., *Brit. J. Exp. Path.,* 1953, v34, 412.

Received December 22, 1953. P.S.E.B.M., 1954, v85.

Effect of Cortisone on Serum Calcium, Magnesium and Phosphate Levels in Nephrectomized Dogs. (20958)

ARTHUR GROLLMAN.

From the Department of Experimental Medicine, Southwestern Medical School, University of Texas, Dallas.

During the course of studies on the electrolyte exchange between the tissues and extracellular fluid after nephrectomy, it was noted that the survival of nephrectomized dogs was shortened by the injection of cortisone (5 mg per kilo of body weight daily) and that such animals showed the cardiotoxic and other effects of hypercalcemia(1). This was confirmed by analyses of the blood which revealed marked increases in the calcium of the serum to toxic levels.

Methods. Mongrel dogs were nephrectomized in two stages with an interval of at least two weeks between the operations. Following nephrectomy the animals were maintained on an electrolyte-free, fat and carbohydrate diet with an amount of water (25 ml/kg of body weight) to compensate for the insensible perspiration. By this procedure the survival period of the animals in good condition is prolonged for periods of 4 to 7 days without vomiting. Serum calcium was determined by Clark and Collip's modification of the Kramer-Tisdall method(2); magnesium by Hawk, Oser and Summerson's modification of Denis' method(3) and inorganic phosphate by the method of Fiske and SubbaRow(4). The Coleman Universal Spectrophotometer was used for all colorimetric comparisons.

The *results* of typical experiments are reproduced in Table I. The values for the serum calcium, inorganic phosphate and magnesium of five dogs on the day following nephrectomy are compared with the values noted 3 or 4 days later during which time they received injections of 50 or 100 mg of cortisone intramuscularly, daily.

TABLE I. Effect of Cortisone on Serum Calcium, Inorganic Phosphorus and Magnesium Levels of the Nephrectomized Dog. All values are expressed as mg/100 ml of serum.

	Calcium	Phosphate	Magnesium
Control	10.8	7.6	2.0
After 100 mg cortisone for 3 days	14.9	13.6	2.8
24 hr later	11.5	16.8	3.5
Control	11.0	6.0	1.9
After 50 mg cortisone for 4 days	15.9	14.7	3.1
24 hr later	12.0	16.0	3.0
Control	8.8	9.9	2.0
After 50 mg cortisone for 3 days	13.7	13.5	2.3
Control	10.3	8.4	2.1
After 50 mg cortisone for 3 days	13.3	13.6	2.5
Control	10.8	6.8	1.8
After 50 mg cortisone for 4 days	15.4	15.2	3.0

Discussion. It is well known that cortisone induces a negative calcium balance which is reflected in the osteoporosis observed in patients suffering from Cushing's disease. However, the level of the serum calcium in the presence of normal renal function, remains unaffected by the administration of cortisone, the calcium mobilized from the blood being excreted in the urine and to a large extent into the gut(5,6). In the presence of normal renal function there may actually be a tendency towards hypocalcemia in the presence of hypoparathyroidism(6). In the nephrectomized dog, on the other hand, where renal excretion is impossible and where excretion through the gut may perhaps also be decreased, the mobilized calcium increases and may reach toxic levels as shown in Table I. Studies on nephrectomized dogs not receiving cortisone reveal a slight rise in serum calcium on the day following nephrectomy with a slight progressive decline thereafter.

The present findings are not without practical interest since they indicate the need for taking precautions in the use of cortisone (and presumably of ACTH) in the face of renal insufficiency to avoid the toxic effects of hypercalcemia.

Summary. The injection of cortisone in the nephrectomized dog increases the serum calcium to toxic levels despite the concomitant rise in serum inorganic phosphate and magnesium. On cessation of therapy the serum calcium level declines again to normal levels.

1. Grollman, A., *Pharmacology and Therapeutics*, 2nd ed., Lea & Febiger, Philadelphia, 1954.

2. Clark, E. P., and Collip, J. B., *J. Biol. Chem.*, 1925, v63, 461.

3. Hawk, P. B., Oser, B. L., and Sommerson, W. N., *Practical Physiological Chemistry*, 12th ed., The Blakiston Co., Philadelphia, 1947.

4. Fiske, C. H., and SubbaRow, Y., *J. Biol. Chem.*, 1925, v66, 375.

5. Soffer, L. J., Gabrilove, J. L., and Jailer, J. W., *J. Clin. Endocrinol.*, 1950, v10, 594.

6. Moehlig, R. C., and Steinbach, A. L., *J. Am. Med. Assn.*, 1954, v154, 42.

Received January 19, 1954. P.S.E.B.M., 1954, v85.

Changes in Patterns of Secretion of Corticosteroids in Rabbits after Prolonged Treatment with ACTH.* (20959)

E. H. Kass, O. Hechter, I. A. Macchi, and T. W. Mou.
(Introduced by M. Finland.)

From the Thorndike Memorial Laboratory, Second and Fourth Medical Services (Harvard), Boston City Hospital, the Department of Medicine, Harvard Medical School and the Worcester Foundation for Experimental Biology, Shrewsbury, Mass.

The nature of the adrenocortical secretion in diverse species has now been evaluated *in vivo* and *in vitro*. It was first shown that the isolated bovine gland, perfused with ACTH, synthesizes a mixture of corticosteroids, the major components of which are 17-hydroxycorticosterone (hydrocortisone) and corticosterone(1). Subsequent examination of the steroids present in adrenal vein blood has revealed that either hydrocortisone or corticosterone (or a mixture of both) is uniformly present in highest concentration in all species studied(2). The relative amounts of hydrocortisone and corticosterone, however, vary widely among species. It has been reported that the adrenal glands of sheep and monkeys secrete primarily hydrocortisone, those of rabbits and rats produce predominately corticosterone, while cows, ferrets, cats and dogs secrete mixtures of both(2,3). Recent studies indicate that human adrenal vein blood contains primarily hydrocortisone, although lesser amounts of corticosterone have been detected(4). These differences between species have been reported by Bush(2,3) to be constant and unaltered by ACTH; the inference has been drawn that the species differences may be fixed genetically.

The view that the nature of the steroids released in response to ACTH is fixed in unalterable fashion seemed at variance with certain observations on the comparative effects of ACTH and corticosteroids on lymphoid tissues of the rabbit. Thus, ACTH, cortisone and hydrocortisone induced significant changes in the weight and concentrations of ribonucleic acid in lymph nodes. Corticosterone, on the other hand, the presumed product of adrenal secretion in rabbits, had no significant effect at the same dosage levels as the other 2 steroids(5,6). The response of the rabbits to ACTH was not explicable on the assumption that rabbits secrete corticosterone exclusively. Inasmuch as the effects of ACTH on lymph nodes were observed after relatively prolonged administration, the possibility was considered that prolonging the treatment with ACTH might alter the pattern of secretion of corticosteroids in this species. Accordingly, experiments were undertaken to test this view. The results to be reported demonstrate that the prolonged administration of ACTH to the rabbit leads to an alteration in the pattern of secretion of corticosteroids so that hydrocortisone becomes the predominant steroid component of adrenal vein blood.

Methods. White male rabbits weighing between 2.5 and 3.5 kg, with no apparent dis-

---

* Aided by grants from the U. S. Public Health Service and the Research and Development Division, Office of the Surgeon General, Department of the Army.

ease, were used in these experiments. To obtain sufficient corticosteroid for analysis, adrenal vein blood from identically treated rabbits was pooled and analyzed as a single sample. Four groups of rabbits were studied. Group I represented untreated rabbits; the other groups received porcine ACTH as follows: Group II received 15 I.U. of ACTH in gelatin† intramuscularly 2-3 hours before collection of the adrenal vein blood was begun. Groups III and IV received ACTH daily for 7 days and 21-28 days, respectively. For the latter groups, a highly purified ACTH preparation (25-50 I.U. per mg as evaluated by bioassay in mice(7)) was suspended as a dry powder in sterile peanut oil containing 5% beeswax. This suspension contained one mg per ml of highly purified ACTH, and was given intramuscularly daily in 0.5 ml doses for the indicated period of time. The prolonged treatment with this preparation of ACTH produced significant loss of body weight and marked hyperplasia of the adrenal glands. The adrenal weights were approximately doubled after 7 days of treatment and approximately tripled after 21-28 days of treatment. The adrenal vein blood was collected from a segment of the vena cava so isolated that only blood from the left adrenal vein could enter it. The left adrenal vein could not be cannulated directly inasmuch as it runs its course almost entirely within the adrenal cortex, entering the renal vein almost at the junction of the left renal vein and inferior vena cava. The right adrenal gland was technically more difficult to isolate in this manner, hence the left gland was used exclusively in these studies. To achieve the vena caval pouch for the collection of adrenal vein blood, the animals were anesthetized with intravenous sodium pentobarbital. A longitudinal midline incision was made over the abdomen, and the inferior vena cava and peritoneal surfaces of the left kidney and left adrenal gland were exposed. Double ligatures were placed around the renal vein distal to the adrenal gland and around the vena cava above and below the left renal vein. All tributaries entering the pouch were ligated. Ligation of the vena cava was the last procedure immediately preceding the opening of the pouch and onset of collection of blood through a glass cannula using mild suction. An average of 4-6 ml of blood per hour was obtained in this manner; the oozing of blood from the adrenal vein was visualized directly in all cases. Five percent glucose in physiological saline was administered through the femoral vein at a rate of 1-2 ml per minute in order to retard the development of circulatory collapse. Thrombosis in the adrenal vein was minimized by the intravenous administration of 10 mg of heparin, and sufficient additional heparin was added to the glucose and saline to deliver about 10 mg per hour. Collection periods extended for 2-3 hours. The samples of adrenal vein blood were pooled upon collection and stored at —20°C. The corticosteroids were extracted from the thawed blood with ethyl acetate, using the method of Meyer(8). The residues obtained from the ethyl acetate extracts were fractionated between 80% ethanol and hexane. The hexane was discarded and the corticosteroids were obtained from the aqueous ethanolic solution. The ethanol was removed *in vacuo* under nitrogen and the corticosteroids were extracted from the aqueous fraction with methylene chloride. The residues obtained after evaporation were fractionated by paper chromatography using the chloroform-formamide and toluene-propylene glycol systems of Zaffaroni(9), using 2 cm wide paper strips for each sample. The corticosteroids present on papergrams were detected by ultraviolet scanning and by reaction of narrow strips to a variety of spot test reagents which included blue tetrazolium and concentrated sulfuric acid. For quantitative estimation of the steroid content, the steroid zone was eluted from paper with methanol, and the ketol content of aliquots of the residue was determined using the procedure of Chen and Tewell(10), using both hydrocortisone and corticosterone as standards. In all cases an area of paper derived from the same papergram, not containing steroid, was eluted and analyzed to obtain a "paper blank." Recovery by this method of corticosteroids

---

† All the ACTH was kindly supplied by Armour Laboratories.

TABLE I. Effect of Administration of ACTH on Concentrations of Corticosterone and Hydrocortisone on Adrenal Vein Blood of 4 Groups of Rabbits.

No. rabbits	Treatment	Vol. sample (ml)	H†	C†	Ratio H/C
			μg/100 ml		
6	0 (control)	69*	<6–8	155	<.05
	ACTH				
5	Single dose	65	<6–8	140	<.05
4	7 days	50	62	126	.5
5	21–28 days	60	307	75	4.

* Estimated value: Pool of 45 ml of adrenal vein blood plus 75 ml from 1 rabbit consisting mainly of extra-adrenal blood.

† H = hydrocortisone; C = corticosterone.

added to blood is 80-100%. Corticosterone and hydrocortisone were identified on the basis of comparison with reference standards, mobility in paper chromatographic systems, the green-yellow fluorescence on paper strips with concentrated sulfuric acid, mixed chromatograms and by the sulfuric acid chromogen technic of Zaffaroni(9).

Results. The results obtained are in Table I. The adrenal vein blood from untreated rabbits contained corticosterone but no detectable hydrocortisone. Since the method employed would have detected 6-8 μg of steroid per 100 ml of blood with ease, the ratio of hydrocortisone to corticosterone is <0.05, in agreement with the findings of Bush(3). When ACTH was given once as 15 I.U. in a gelatin menstruum, the results were similar. This suggests that the considerable trauma associated with the collection procedure may have stimulated the release of endogenous ACTH at rates sufficient to stimulate biosynthesis of corticosteroids at or near maximal capacity. Similar observations following brief treatment with ACTH have been reported(3).

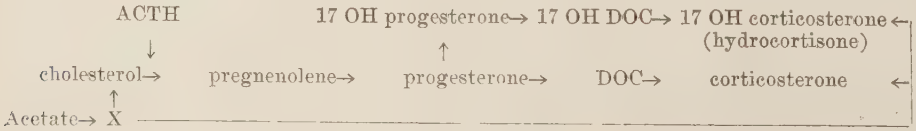
When daily treatment with ACTH was given for one week or more, hydrocortisone appeared in the adrenocortical secretion. After 7 days of treatment, the ratio of hydrocortisone to corticosterone was about 0.5; after 21-28 days hydrocortisone became the predominant steroid and its concentration was 4 times that of corticosterone. There is an indication that the total corticosteroid output was increased in this circumstance, perhaps as a reflection of the increase in adrenal weight. Evaluation of the absolute rate of output of corticosteroid could not be attained precisely due to variations in rates of blood flow, adrenal weight, collection periods, etc. However, a general figure of 40-75 μg of steroid per g of gland per hour represents the approximate magnitude of the rate of production. No clear effect of ACTH, regardless of the manner of administration, on the rate of production of steroid per g of adrenal tissue per unit time could be ascertained.

All of the papergrams of the adrenal vein samples contained, in addition to the 2 identified components, zones of unidentifiable material giving ultraviolet reactions typical for Δ^4-3 ketones of varying polarity. No 17-ketosteroid reaction zones were detected. Those ultraviolet reactive zones that did not react with blue tetrazolium may have represented adrenal products since they were not present in detectable amounts in extracts of equivalent volumes of peripheral rabbit blood. In general, all samples studied, with the exception of those obtained after ACTH had been given for 3-4 weeks, showed the presence of 2 such zones, one more polar than hydrocortisone and the other of polarity similar to those of cortisone and electrocortin. After 3-4 weeks of administration of ACTH, 3 ultraviolet positive zones more polar than hydrocortisone and 3 zones of polarity intermediate between hydrocortisone and corticosterone were observed. Whatever the nature of these unidentified substances, the suggestion from these studies is that the administration of ACTH for 3-4 weeks may also influence the number or concentration of these unidentified trace components. Although present in minor concentration, these trace products are not necessarily without physiological interest; a trace component of the adrenocortical secretion, electrocortin, has recently been described and may be primarily responsible for the adrenal control of electrolyte metabolism (11,12).

Discussion. These data may serve to explain why ACTH and hydrocortisone exerted similar effects on the lymph nodes of rabbits when corticosterone in large doses was without significant effect. The treatment with ACTH in the lymph node studies was administered for 5 days or more(5,6). Our present results indicate that significant amounts of hydrocortisone were probably produced by the stimulated adrenal glands. There are indications that certain effects of ACTH are different in different animal species, presumably through variable effects on adrenal steroidogenesis. These data have been reviewed elsewhere(7,13).

Although genetic factors are undoubtedly operative in the control of adrenal secretions, the results herein reported demonstrate that the nature of the adrenal secretion within a given species is not unalterably fixed. Whether factors other than exogenous ACTH have a similar effect on the adrenal secretion remains for future investigation. It has been demonstrated, *in vitro,* that exogenous ACTH rapidly increases the rate of production and release of corticosteroids without significant alteration of the nature of the steroids produced(3,14,15). The effect of ACTH described in these studies is dissimilar in that it requires days or weeks to achieve the profound alteration in the pattern of adrenal secretion without striking increase in the rate of production of corticosteroid. The rapid action of ACTH has been extensively studied *in vitro;* this action appears to be involved in the conversion of cholesterol to progesterone from which both hydrocortisone and corticosterone are derived(15,16). In confirmation of these *in vitro* observations Bush has found that ACTH increases the total steroid output without altering the relative ratios of hydrocortisone to corticosterone under certain conditions(3).

The action of ACTH which is demonstrable after prolonged treatment must operate differently in that the pattern of steroids but not necessarily the absolute amount is altered. The following sequence in the biosynthesis of corticosteroids from cholesterol *in vitro* has been proposed(16), and the locus of action of ACTH is indicated:



From this sequence it is apparent that the rate of hydroxylation of progesterone at C-17 and C-21 is a determining factor in the synthesis of hydrocortisone and corticosterone and that synthesis of corticosterone is the major pathway of conversion of progesterone if C-17 hydroxylating activity is low or absent. If this hypothesis is correct, the absence of secretion of hydrocortisone in untreated rabbits would be due to a deficiency or inhibition of the C-17 hydroxylating system involved in biosynthesis of hydrocortisone. Conversely, according to this view, the appearance of hydrocortisone in the adrenal secretion requires that prolonged treatment with ACTH selectively increase the activity of the C-17 hydroxylating system. Further work is clearly necessary to test this hypothesis.

Summary. The adrenal vein blood of untreated rabbits contains corticosterone as the predominant steroid component. A single dose of ACTH did not alter significantly the nature or amount of steroid output of the adrenal gland. When ACTH of porcine origin was given for one week, hydrocortisone as well as corticosterone was found in the adrenal vein blood in a ratio of 1:2. The administration of ACTH for 21-28 days led to the predominance of hydrocortisone in the adrenal secretion, and the ratio of hydrocortisone to corticosterone was 4:1.

The authors are grateful to Dr. Robert R. Aycock and to Miss Marguerite M. Lundgren for much helpful assistance.

---

1. Hechter, O., *et al., Recent Progress Hormone Research,* 1951, v6, 215.

2. Bush, I. E., *J. Endocrinol.,* 1953, v9, 95.

3. ———, *Ciba Foundation Colloquia on Endocrinology,* 1953, v7, 210, Churchill, London.

4. Romanoff, E. B., Hudson, P., and Pincus, G., *J. Clin. Endocrinol.,* 1953, v13, 1546.

5. Kass, E. H., and Kendrick, M. I., *Fed. Proc.,* 1952, v11, 472.

6. Kass, E. H., Kendrick, M. I., and Finland, M., *Ann. New York Acad. Sc.,* 1953, v56, 737.

7. Kass, E. H., Lundgren, M. M., and Finland, M.,

J. Exp. Med., 1954, v99, 89.

8. Meyer, A. S., *J. Biol. Chem.*, 1952, v203, 469.
9. Zaffaroni, A., *Recent Progress Hormone Research*, 1953, v8, 51.
10. Chen, C., Wheeler, J., and Tewell, H. E., Jr., *J. Lab. and Clin. Med.*, 1953, v42, 749.
11. Simpson, S. A., *et al.*, *Experientia*, 1953, v9, 333.
12. Simpson, S. A., Tait, J. F., and Bush, I. E., *Lancet*, 1952, v263, 226.
13. Kass, E. H., and Finland, M., *Ann. Rev. Microbiol.*, 1953, v7, 361.
14. Hechter, O., *Ciba Foundation Coloquia on Endocrinology*, 1953, v7, 161, Churchill, London.
15. ———, Trans. 3rd Conf. Adrenal Cortex. Macy Foundation, Ed. E. Ralli, 1952, p. 115.
16. Stone, D., and Hechter, O., *Abstr. XIX Int. Physiol. Congress* (Montreal), 1953, p. 805.

Received February 19, 1954. P.S.E.B.M., 1954, v85.

Changes in Clot Retraction Time and Platelet Adhesiveness Following Whole Body Irradiation.* (20960)

J. PHILIP SAVITSKY AND SOL SHERRY.

From the May Institute for Medical Research of the Jewish Hospital Association, Cincinnati, O.

The exact nature of the clotting defect in the hemorrhagic state following whole body radiation is unclear. It is apparent that the concentrations of fibrinogen(1), calcium, prothrombin(1), ac-globulin(2), antihemophilic globulin(3) and the plasma precursor of serum prothrombin conversion accelerator(4) are normal in the irradiated animal. The most significant concomitant of the prolonged clotting time and the hemorrhagic syndrome is the marked thrombocytopenia. Emphasizing the significance of the thrombocytopenia have been the studies demonstrating temporary reversal of the clotting defect following transfusion into these animals of normal platelets (5,6). It is not known whether there are qualitative changes in platelets of irradiated animals or whether these changes may precede onset of thrombocytopenia. Cohn has shown in his charts that there is a delay in initiation of clot retraction by the third post radiation day prior to onset of thrombocytopenia but no reference is made to this observation in the text(7). There is a very close relation between the rate of initiation of clot retraction as measured by clot retraction time and level of platelet adhesiveness(8,9).

In this study evidence is presented of changes in platelet adhesiveness and clot retraction time preceding onset of thrombocytopenia in the irradiated dog. There is also present in the irradiated animal a circulating plasma factor which inhibits platelet adhesiveness and prolongs the initiation of clot retraction.

Methods. Citrated venous blood was placed in a lusteroid tube and immediately after drawing blood a sample was drawn in a red-cell diluting pipette. The diluting fluid was 3.8% sodium citrate and 0.2% formalin. The pipette was placed in shaker for 10 minutes and the fluid then examined directly on a counting chamber. Platelet adhesive index was determined by the glass-wool method of Moolten and Vroman(10). The normal index in the dog varied from 1.15 to 1.35 and is reproducible with less than 10% error. Clot retraction time was determined by the method of recalcified venous blood in castor oil(8). This was modified to account for the more rapid clotting of recalcified dog's blood. 4.5 ml of venous blood was drawn into a syringe containing 0.5 ml of 3.8% sodium citrate. One ml of this blood was placed in each of 2 glass tubes in a water bath at 18°C and .06 ml of a 5% $CaCl_2 \cdot 2H_2O$ solution was added to each tube. Immediately following addition of calcium, blood was drawn up in a siliconized capillary pipette and deposited as a single drop in castor oil. The tubes of castor oil were kept at 18°C and the test done in quadruplicate. The clot retraction time was

---

* This investigation was done under Contract with the U. S. Atomic Energy Commission.

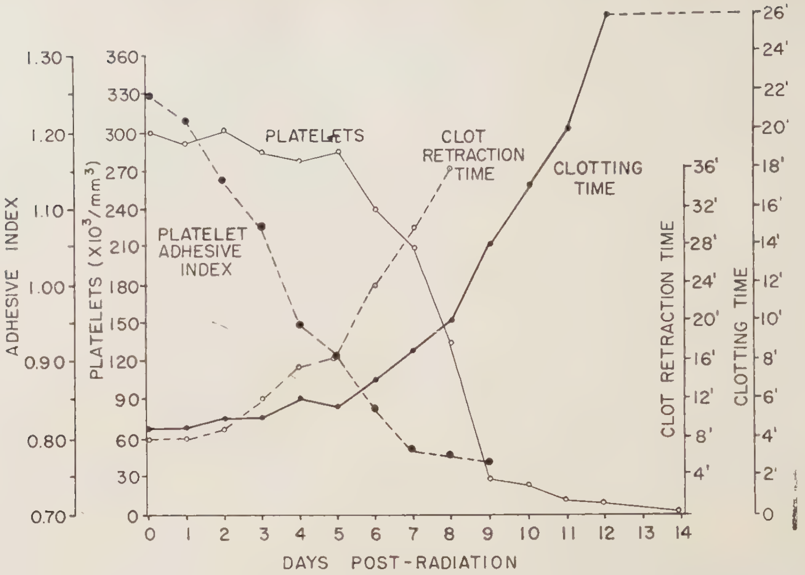


FIG. 1. Average daily values of platelet adhesive index, platelet count, clot retraction time and clotting time of 8 dogs following 400 r whole body radiation.

the time from clotting of recalcified blood to exudation of serum from the drop in castor oil. The normal range, as performed on 15 dogs, was 6-10 minutes, mean value 7.5 minutes. Clotting time was done in a glass clotting tube at room temperature. Blood was drawn using 2-syringe technic. Normal clotting time in dogs varied from 3 to 6 minutes. Dogs weighing 20 to 30 lb received 400 r, 250 kv. peak, 15 ma, dose rate 25 r/min. Target skin distance, 100 cm. Dosage of 400 roentgens was LD_{90}.

Results. Eight healthy dogs were given 400 r of whole body radiation and studied daily until time of death. Daily determinations were made of platelet count, platelet adhesive index, clot retraction time and clotting time. Results are shown in Fig. I. There was a significant decrease in platelet adhesiveness by the third and fourth post radiation days and a concomitant prolongation of clot retraction time. These changes occurred prior to any demonstrable changes in platelet count or clotting time. When the platelet count dropped below 100000/mm³, clot retraction did not occur and it was difficult to determine platelet adhesive index.

It was observed accidentally that *in vitro* addition of excess calcium to blood from an irradiated dog returned a prolonged clot retraction time to normal. The effect on clot retraction time, of varying the concentration of calcium was then determined. Different

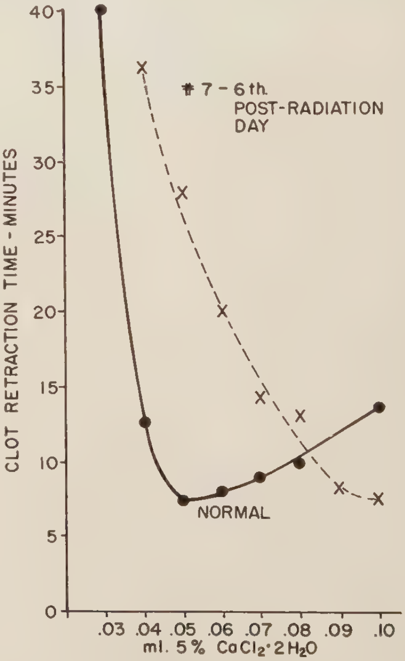


FIG. 2. Effect of varying concentrations of calcium on clot retraction time of normal and irradiated dogs blood.

TABLE I. Inhibition of Clot Retraction and Platelet Adhesiveness by Plasma from an Irradiated Dog.

	Clot retraction time, min.	Platelet adhesive index
1 cc N*	7	1.23
Idem + .1 cc citrate-saline	6	—
" .2 *idem*	8	1.26
" .1 normal plasma	6	—
" .2 *idem*	7	1.22
" .1 plasma (irrad.)	8	—
" .2 *idem*	18	.87

* N = Normal blood.

amounts of calcium chloride solutions were added *in vitro* to normal dog's citrated blood and to citrated blood from irradiated animals. Blood from irradiated dogs was drawn on 5th to 7th days post radiation when they had a markedly prolonged clot retraction time but with at least 50% of the platelets still present. The results of a typical experiment are shown in Fig. 2. The curve for normal blood is similar to that observed for coagulation time with variation in calcium concentration. The shift of the clot retraction time curve in the irradiated animals to a higher concentration of calcium suggests a defect in the use of calcium in initiation of clot retraction. The calcium concentration in blood when 0.1 ml of a 5% $CaCl_2$ solution is added, is far in excess of physiological concentration. These results suggested that not only was there a defect in the platelet prior to onset of thrombocytopenia but that perhaps there might be a substance present in plasma of these irradiated animals which damaged the platelets and inhibited initiation of clot retraction. This was tested by adding plasma from a thrombocytopenic dog (12th post radiation day) to normal blood *in vitro*. To one ml of citrated blood from a normal dog, 0.2 ml of thrombocytopenic plasma and 0.7 cc of 5% $CaCl_2$ solution were added. This was found to prolong clot retraction time of normal blood whereas addition of normal plasma to normal blood had no effect. A typical experiment is shown in Table I. Further studies showed that this prolongation of clot retraction time of normal blood could be prevented by addition of excess calcium with a similar shift of the curve to higher concentrations of calcium as shown in Fig. 2. Also plasma from irradiated dogs markedly decreased platelet adhesiveness of normal blood in the same proportions of added plasma to normal blood (Table I).

The properties of this clot retraction inhibitor present in plasma of irradiated dogs were investigated. It was found to be non-dialyzable, water soluble, stable at 4°C for several weeks, stable at 56°C for at least 30 minutes and at 60°C for at least 20 minutes. This substance was destroyed in 5 minutes at 65°C. It was stable over a pH range of 5-9 and was not precipitated with globulins at pH 5.2 (diluted with distilled water). It was not adsorbed by $BaSO_4$, $Ca_3(PO_4)_2$ or $Al(OH)_3$. All attempts at extraction with lipid solvents were unsuccessful.

The inhibitor of the initiation of clot retraction and of platelet adhesiveness was not demonstrable in the plasma of the irradiated dog until 8th post radiation day. It was most easily shown when the animal became thrombocytopenic at about 10th to 12th post radiation days, although it was never possible to add less than 0.2 cc of plasma to one ml normal blood and produce inhibition of clot retraction time.

Discussion. Our data indicate that qualitative changes in platelets occur prior to development of thrombocytopenia associated with radiation hemorrhage. Concomitant with the decrease in platelet adhesiveness is a prolongation of clot retraction time, which is a measure of the rate of initiation of clot retraction. Platelets start to disappear from circulation by 5th to 6th post radiation days at a time when platelet adhesive index is extremely low and clot retraction time markedly prolonged. It may be that changes in the surface properties of the platelets play some role in subsequent rapidly developing thrombocytopenia.

There is present in the plasma of the irradiated dog, a substance which lowers the platelet adhesiveness and inhibits clot retraction time of normal blood. The demonstration of a circulating factor present 8 to 15 days following radiation which can induce changes in normal blood similar to those occurring in radiated animal raises again the question of the "indirect" effect of radiation. The extensive investigations of Lawrence and his coworkers(11) of lack of effect of cross transfusions from radiated animals into normal have provided strong negative evidence. These studies have been done *in vivo,* whereas the evidence presented here for a plasma clot retraction time inhibitor has been entirely *in vitro.* The inability to demonstrate this factor before the 8th post radiation day is significant because the studies of Lawrence *et al.* extended only through the 5th post radiation day.

Conclusions. 1. There is an inhibition of platelet adhesiveness and a prolongation of clot retraction time following whole body radiation of the dog. These changes are demonstrable 3 days after radiation, prior to thrombocytopenia or prolongation of clotting time which is associated with hemorrhage. 2. Inhibition of clot retraction time can be reversed *in vitro* by addition of excesses of calcium. This suggests a defect in the radiated animal in the use of calcium in initiation of clot retraction. 3. There is a substance in the plasma of the radiated dog which acts on platelets to decrease platelet adhesiveness and inhibit clot retraction time. This factor is not demonstrable before 8th post radiation day and is most easily shown when the animal is thrombocytopenic.

We wish to thank Mr. Dwight W. Callaway for his excellent technical assistance.

1. Jackson, D. P., Cronkite, E. P., Levoy, G. V., and Halpern, B., *J. Lab. and Clin. Med.,* 1952, v39, 449.

2. Jackson, D. P., Cronkite, E. P., Jacobs, G. J., and Behrens, C. F., *Am. J. Physiol.,* 1952, v169, 208.

3. Penick, G. D., Cronkite, E. P., Godwin, I. D., and Brinkhous, K. M., Proc. Soc. Exp. Biol. and Med., 1951, v78, 732.

4. Jacobs, G. J., and Cronkite, E. P., (Unpublished). Quoted by Cronkite, E. P., and Brecher, G., *Defects in Hemostasis Produced by Whole Body Irradiation. Blood Clotting and Allied Problems,* Trans. 5th Josiah Macy Conference. Josiah Macy Foundation, New York, 1952.

5. Brecher, G., and Cronkite, E. P., *Proc. New York Path. Soc.,* 1950-1951, v116.

6. Woods, M. C., Gamble, F. N., Furth, J., and Bigelow, R. R., *Blood,* 1953, v8, 545.

7. Cohn, S. H., *ibid.,* 1952, v7, 225.

8. Savitsky, J. P., and Werman, R., *Am. J. Clin. Path.,* 1952, v22, 1175.

9. Savitsky, J. P., *Blood,* 1953, v8, 1091.

10. Moolten, S. E., and Vroman, L., *Am. J. Clin. Path.,* 1949, v19, 701.

11. Lawrence, J. S., Valentine, W. N., and Dowdy, A. H., *Blood,* 1948, v3, 593.

Received February 23, 1954. P.S.E.B.M., 1954, v85.

Production of Renal Hypertension by Injection of Finely Divided Silica into Renal Artery.* (20961)

Campbell Moses.

From the Addison H. Gibson Laboratory, University of Pittsburgh School of Medicine.

The frequent occurrence of sustained hypertension in the course of chronic renal disease and the need for a procedure to produce experimental hypertension by an intrarenal lesion occasioned this study. The development of marked fibrosis as a reaction to silica in the liver(1-3) indicated that a similar technic applied to the kidney might produce sustained hypertension.

Methods. Mongrel dogs prepared with an exteriorized carotid artery to permit the ready determination of arterial systolic pressure in the unanesthetized state were kept in the laboratory under uniform conditions. The systolic blood pressure was determined by palpation of the exteriorized carotid distal to an occluding pneumatic cuff at twice weekly intervals for 10 months. After preliminary trials in 24 dogs had established suitable technic and dosage, 5 dogs anesthetized with

---

* This work was supported in part by research grant from the National Heart Institute of the National Institutes of Health, Public Health Service.

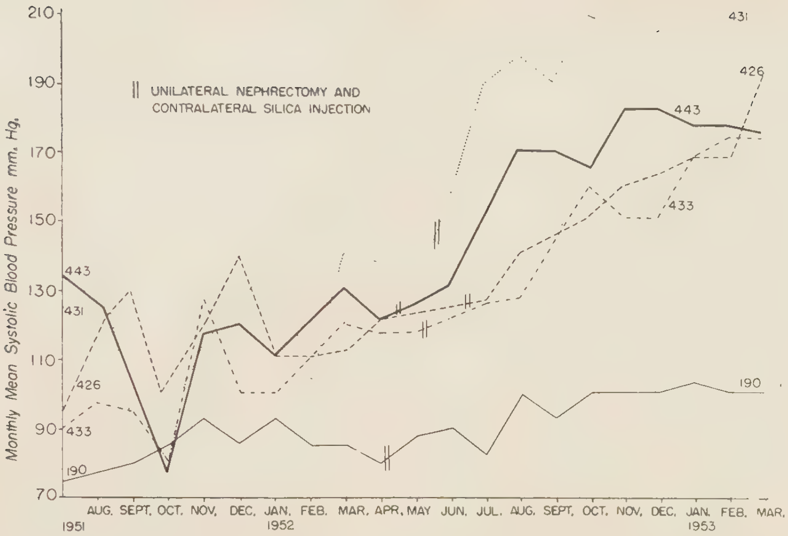


FIG. 1. Monthly mean values for systolic blood pressure in dogs during control period and following unilateral nephrectomy and injection of 5 mg of white silica into the contralateral renal artery.

intravenous pentobarbital were unilaterally nephrectomized, and immediately 5 mg of white silica (0.02 to 0.05 μ) suspended in 2 ml of isotonic saline was injected through a 21-gauge needle into the renal artery of the unoperated kidney as close to the renal pelvis as possible. In 4 other dogs the renal arteries of both kidneys were similarly injected at one operation. Care was taken to have the syringe and needles meticulously clean and the silica suspension freshly made to prevent the jamming of silica in the syringe. Bleeding from the site of the arterial puncture was prevented by finger pressure. In the control and immediate postoperative periods blood urea nitrogen levels were obtained at frequent intervals. After recovery from the operative procedure, the weekly or biweekly blood pressure determinations were resumed. At no time did the observer recording the blood pressure have information as to the operative procedure carried out in the animal under study.

Results. Fig. 1 records the mean values of the systolic pressure that were obtained during each month of this experiment. Fig. 2 records similarly obtained monthly mean values of the systolic pressures in four additional dogs in whom 5 mg of white silica suspended in 2 ml of saline was injected into the renal arteries of both kidneys at the time of laparotomy.

The blood urea nitrogen levels in all animals were normal prior to the silica injection. In the animals subjected to silica injection and contralateral nephrectomy, there was a prompt elevation of the blood urea nitrogen to 35-50 mg % during the postoperative period, but this returned to preoperative levels within 3 weeks. In the animals receiving bilateral renal artery injections a gradual elevation of blood urea nitrogen occurred, reaching a maximum of about 50 mg % at 4 weeks postoperatively and returning to preoperative levels within 8 to 10 weeks.

During the preliminary experimental trials, death from uremia occurred in several animals when 10 or more mg of silica in suspension was injected. With doses of 20 or more mg anuria with uremia and death in 72 hours frequently occurred.

All dogs in this study were sacrificed 8 to 12 months after silica injection. The kidneys injected with silica appeared normal externally. On hemisection there appeared to be diffuse fibrosis of the cut surface with increased prominence of the glomeruli. No hemorrhages were noted. The microscopic sections from all dogs including the 2 that failed to develop hypertension revealed a

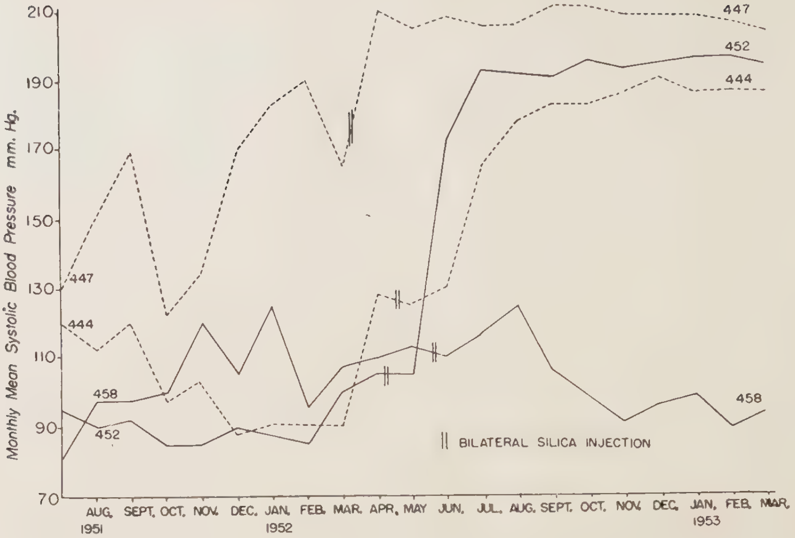


FIG. 2. Monthly mean values for systolic blood pressures in dogs during control period and following injection of 5 mg of white silica into both renal arteries.

diffuse fibrous tissue reaction in the glomeruli with a considerable reduction in the number of functioning units. The tubules were usually of normal appearance, but in a few patchy areas they were replaced by diffuse fibrosis.

Discussion. The wide spontaneous variations in dog blood pressure as determined without anesthesia using the exteriorized carotid (Fig. 1, 2) emphasized the need for an adequate control period in this study. The sustained elevation of arterial pressure, developing in 4 of the 5 unilaterally nephrectomized dogs receiving silica and in 3 of the 4 dogs with bilateral silica injection, indicates that this method may be useful as another method for producing sustained hypertension. The lesion produced by this technic is primarily an intrarenal one and therefore is in some respects similar to the situation in clinical hypertension due to chronic renal disease.

Explanation for the failure of one animal in each group here reported to develop hypertension is not available from this study. The elevation of blood urea nitrogen occurring in these 2 animals was similar to that observed in those developing hypertension, and additional study with determination of renal blood flow, glomerular filtration rate, etc. will be necessary for understanding of the mechanisms involved in this reaction.

Summary. A method for the production of experimental hypertension by the injection of 5 mg of white silica into the renal artery is described. The intrarenal fibrous tissue reaction occurring in response to this injection is apparently responsible for the elevation of blood pressure that occurs.

The white silica used was made by the Linde Laboratories, Tonawanda, N. Y., and provided through the courtesy of Dr. Charles P. Carpenter of the Mellon Institute of Industrial Research.

---

1. Gye, W. E., and Purdy, W. J., *British J. Exp. Path.*, 1922, v3, 94.

2. Gardner, L. U., and Cummings, D. E., *Am. J. Path.*, 1933, v9, 751.

3. Rousselat, L. M., and Thompson, W. P., PROC. SOC. EXP. BIOL. AND MED., 1939, v40, 705.

Received March 1, 1954. P.S.E.B.M., 1954, v85.

Reduction of Serum Complement Following *in vivo* Tissue Antigen-Antibody Reactions. (20962)

A. B. STAVITSKY, D. B. HACKEL, AND W. HEYMANN.

From the Departments of Microbiology, Pathology and Pediatrics, Western Reserve University School of Medicine and City Hospital, Cleveland, O.

In a previous study(1) serum complement activity was found to be reduced following the interaction of *circulating* antibody and *injected* antigen in the rabbit. Two types of evidence pointed to the requirement of circulating antibody for the reduction of complement. First, 2 injections of antigens about 90 minutes apart into an immunized rabbit apparently bound all of the circulating antibody so that a third injection of antigen failed to produce a decrease of complement(1). Second, when circulating antibody was allowed to disappear without further injection of antigen, subsequent injections of antigen did not bring about a decrease of complement(1). However, these experiments did not eliminate the possibility that *tissue* antigen-antibody reactions, where either antigen and antibody or both were associated with the tissues, could also cause the depression of complement *in vivo*. There have been reports(2-4) of the lowering of complement in connection with cytotoxic or allergic manifestations *in vivo* which probably involved tissue antigen-antibody reactions. Therefore, it was of interest to ascertain the effect on complement of the injection of rabbit anti-rat kidney serum into rats. Such injections are known to produce a rapid reaction between tissue antigens in the kidney, liver and lungs and corresponding antibodies in the antikidney serum(5). This reaction results in a renal disease which simulates the nephrotic syndome as it is observed in infants and children(6).

Materials and methods. Animals. Sprague-Dawley rats of both sexes weighing about 130-160 g were employed. They were fed a diet of Friskies and water. *Preparation of antisera.* Antikidney serum was prepared in rabbits by intraperitoneal injections for several weeks of a 20% homogenate in saline of perfused whole rat kidney(6). It was stored at −20°C. In the course of the work the possibility arose that complement was being fixed as a result of reactions between rat plasma and antibodies to rat plasma in the rabbit antiserum. Therefore, aliquots of antikidney serum were absorbed with rat plasma to remove corresponding antibodies. In order to try to define the role of the kidney antigen-antibody system sera were also absorbed with rat kidney. 0.3 ml of a 50% rat kidney homogenate was lyophilized in a narrow tube. 0.3 ml of rat plasma was pipetted into a test tube and lyophilized. For absorption 0.25 ml of antikidney serum was placed in a tube containing either lyophilized kidney or plasma and the dried kidney or plasma resuspended or redissolved in the serum. After 1 hour's incubation at 37°C it was considered that the plasma antibodies had been removed and the contents of the tube were called "absorbed antiserum." Tests described below indicated that the plasma antibodies had been removed completely by this procedure. There was no suitable method for determining *in vitro* whether the kidney antibodies had been removed. Antibodies to rat red cells were removed by absorption of the antikidney serum with an equal volume of washed rat red cells. *Estimation of antibody.* Antibody to rat plasma was estimated by the *ring reaction.* Rabbit antikidney serum was placed in a capillary tube and overlaid with an equal volume of rat plasma. The appearance of an interphasic precipitate indicated a positive reaction. However, a much more sensitive method for detecting this antibody was provided by the *hemagglutination reaction* as modified in one of our laboratories (A.B.S.)(7) from Boyden(8). Red blood cells were treated with tannic acid after which they adsorbed rat plasma on their surface. These tannic acid-plasma cells were then agglutinated by antibodies to rat plasma. By this method rat plasma antibodies have been found consistently in the rabbit anti rat kidney serums. *Assay of complement.* Complement was assayed by the method of Ecker, Hiatt, and Barr(9) in terms of 50% hemolytic units, that is, the number of ml of serum required to lyse 50% of a standard suspension of sensitized sheep red blood cells. *Therefore, the higher the figure the lower the complement concentration of serum and vice versa. Other procedures.* Rats were bled by cardiac puncture. About 0.25 ml of blood was taken each time. Nephrectomies were performed by lumbar approach under ether anesthesia. Nephrectomized animals were fasted and thirsted after the operation. The severity of the renal disease was estimated by quantitative determination of proteinuria, by the presence of anasarca, changes in blood chemistry and histological changes(6). In each experiment the rats were first bled by cardiac puncture. Whereas in previous work(6) the serum was given in divided dosage, in the present study it was given in one dose immediately after the initial bleeding. Thirty minutes and 48 hours after the injection of the serum the animal was bled again. The serum was separated, quick-frozen in CO_2-alcohol and placed in a deep freeze at –20°C until the day of assay. The sera from each experiment were assayed on the same day.

Results. Correlation of complement level and renal disease. Table I shows that the injection of normal rabbit serum into rats was not followed by a reduction of complement. On the other hand, the injection of antikidney serum practically always was followed by depression of complement whether renal disease ensued or not. The failure to produce nephrosis with the injection of antikidney serum was noted (Rats No. 3, 4, 7, 9, 10, 11) and probably due to an insufficient concentration of kidney antibodies in the serum. However, renal disease was not produced in the absence of a reduction of complement.

TABLE I. Serum Complement Levels and Incidence of Renal Disease in Rats after Injection of Control Rabbit Serum, and Anti-Rat Kidney Serum.

Rat		C' at time			Renal
#	Serum #	0	30 min.	48 hr	disease
1	Normal	.9	.85	.80	0
2		.5	.5	.5	0
3	502 AK	.7	1.9	.5	0
4		1.2	4.1	.7	0
5	506 AK	1.7	4.0	2.0	+
6		1.1	4.0	1.7	+
7	493 AK	1.2	1.4	1.2	0
8		.5	3.0	3.0	+
9		.4	.45	.45	0
10	498 AK	1.7	1.7	1.6	0
11		1.6	3.2	3.1	0
12		1.6	3.4		+

C'—ml serum diluted 1:25 required for 50% lysis; AK—anti-rat kidney serum.

TABLE II. Level of Complement after Injection into Rats of Plain Anti-Kidney Serum (AK) or of Anti-Kidney Serum Absorbed with Rat Plasma (AK-RP).

	C' titer at time		
Serum #	0	30 min.	48 hr
517 AK	.4	3.0	1.8
	.6	3.0	—
	.5	3.4	—
517 AK-RP	.5	3.5	.5
	.5	2.8	.8

Complement level following the injection of antikidney serum absorbed with rat plasma or rat red cells. The data in Table I showed that complement was reduced after inoculation of antikidney serum. However, this could have been due to antigen-antibody reactions not involving kidney antigen and antibody. Therefore, the antikidney sera were absorbed with lyophilized rat plasma. Such absorption resulted in reduction of the hemagglutination titer against plasma-coated red cells from 1/163,000 for the unabsorbed serum to 0 for serum absorbed with rat plasma and 1/20,000 for serum absorbed with rat kidney. Since this method will detect fractions of a microgram of antibody protein(7) a negative reaction is significant. The presence of rat plasma antigens in rat kidney preparations of the type used for immunization of rabbits was shown by the great reduction in antiplasma titer caused by absorption of the antikidney serum with lyophilized rat kidney.

Table II presents the results of injections of these absorbed sera into normal rats. It is evident that complement activity was decreased to about the same extent whether the antikidney serum was absorbed or unabsorbed with rat plasma. Complement was also reduced in rats injected with kidney

TABLE III. Reduction of Complement after Injection of Anti-Kidney Serum into Bilaterally Nephrectomized Rats.

Serum #	C′ titer at time 0	C′ titer at time 30 min.
516 AK	.4	2.5
"	1.0	3.0
None	.6	.6
"	.4	.4

antiserum from which rat red cell hemagglutinins were removed completely. *Reduction of complement after the injection of antikidney serum into normal and bilaterally nephrectomized rats.* Conclusive evidence of the participation of extra-renal tissues in the reduction of complement was sought by the injection of antikidney serum into nephrectomized rats (Table III). Complement was reduced in bilaterally nephrectomized rats given this serum whereas bilateral nephrectomy alone did not lead to a loss of complement. Complement was depressed in control rats given this serum and they developed severe renal disease.

Discussion. Several different types of evidence in the present and other studies(5,10) indicate that antikidney serum contains antibodies which react with other components of extra-renal tissues. These cross-reactions appear to be due to the possession by many tissues of common connective tissue antigens associated with basement membranes, reticulum, sarcolemma and neurilemma(10). There is some evidence that these antigens may be mucopolysaccharides(11). The decrease in complement which occurs upon injection of antikidney serum into bilaterally nephrectomized rats shows that the kidney antigens are not absolutely required for this reduction. In fact, no data have been obtained in the present work which definitely implicate the kidney antigen-antibody reaction in the reduction of complement. Indeed there is no definite information on the actual tissues participating, though from recent studies tissues containing common antigens must be involved. Antibodies to rat plasma, however, have been eliminated from participation in this reduction by absorption of the antikidney serum with plasma. Therefore, the presumption is strong that complement was reduced as a result of *tissue* or *cellular* antigen-antibody reactions, excluding red blood cell antigens. Further study will be required to exclude the participation of antigen-antibody reactions between humoral antigens other than those in plasma and corresponding antibodies in the antikidney serum.

The development of the nephrotic syndrome in the rat has always been accompanied by a marked but transient lowering of serum complement. In the human nephrotic syndrome complement was diminished during the active phase of the disease and persisted at low levels for many days or even weeks(12,13). However, even assuming that the lowering of complement in the human disease is associated with an antigen-antibody reaction the situation is not entirely analogous to that in the rat. In the animal the passively conferred kidney or tissue antibodies presumably combine rapidly with the tissue antigens with the reduction of complement. In the human disease the concentration of antibody may be maintained by continued active antibody formation and cause a prolonged antigen-antibody reaction with continued fixation of complement.

In view of these considerations further study is needed to define the role *if any* of complement in the pathogenesis of the experimentally produced nephrotic syndrome of rats as well as other cytotoxic or allergic manifestations of tissue antigen-antibody reactions. Obviously, the reduction of complement which accompanied tissue antigen-antibody reactions in the present study is not incompatible with the participation of complement in these manifestations.

Summary. Serum complement was reduced within 30 minutes after injection of rabbit anti-rat kidney serum into rats. Complement was decreased in amount following the injection of this antiserum into bilaterally nephrectomized rats. Complement also was decreased in rats given this antiserum after antibodies to rat plasma and rat red cells had been removed. Therefore, it was concluded that complement may be reduced *in vivo* as a result of extra-renal tissue antigen-antibody reactions. The significance of the lowering of complement in relation to the

development of the nephrotic syndrome remains to be determined.

The willing and competent technical assistance of Anne Driftmeyer Gardner, Caroling Gilkey and Melena Salehar is gratefully acknowledged.

---

1. Stavitsky, A. B., Stavitsky, R., and Ecker, E. E., *J. Immunol.,* 1949, v63, 389.
2. Kellett, C. E., *Lancet,* 1936, v2, 1262.
3. Ogawa, S., and Sato, Y., *Tr. Soc. path. Jap,* 1938, v28, 212.
4. Schwab, L., Moll, F. C., Hall, T., Breen, H., Kirk, M., Hawn, C. van Z., and Janeway, C. A., *J. Exp. Med.,* 1950, v91, 505.
5. Pressman, D., and Sherman, B.; *J. Immunol.,* 1951, v67, 21.
6. Heymann, W., and Lund, H. Z., *Pediatrics,* 1951, v7, 691.
7. Stavitsky, A. B., *J. Immunol.,* 1954, in press.
8. Boyden, S. V., *J. Exp. Med.,* 1951, v93, 107.
9. Ecker, E. E., Hiatt, C. W., and Barr, L. M., *J. Lab. and Clin. Med.,* 1947, v32, 287
10. Cruickshank, B., and Hill, A. G. S., *J. Path. and Bact.,* 1953, v66, 283.
11. Cole, L. R., Cromartie, W. J., and Watson, D. W., PROC. SOC. EXP. BIOL. AND MED., 1951, v77, 498.
12. Wedgwood, R. J. P., and Janeway, C. A., *Pediatrics,* 1953, v11, 569.
13. Wedgwood, R. J. P., personal communication.

Received March 8, 1954. P.S.E.B.M., 1954, v85.

Effect of Coronary Perfusion of Prostigmine on Ventricular Fibrillation in the Hypothermic Dog.* (20963)

ARTHUR E. PREVEDEL, V. MONTGOMERY, AND HENRY SWAN.
(Introduced by C. A. Maaske.)
From the Halsted Laboratory for Experimental Surgery and the Departments of Surgery and Physiology, University of Colorado School of Medicine, Denver.

Ventricular fibrillation is a frequent event during cardiac surgery with inflow occlusion in the experimental hypothermic dog. Bigelow *et al.*(1), Rosenhain and Penrod(2), and Swan *et al.*(3) have demonstrated that the incidence of ventricular fibrillation increases with body temperatures below 25°C with CO_2 retention and with cardiac manipulation. To minimize the first 2 of these factors in the present work, the body temperature was lowered to the range between 23° and 26°C and the animals were vigorously hyperventilated with pure oxygen sufficiently to maintain a blood pH of about 7.6. A suitable pharmacological agent was then sought to alter the fibrillatory threshold during cardiac manipulation. The drug which significantly altered this fibrillatory threshold was prostigmine.

Methods. Mongrel dogs were placed in an ice water bath after being deeply anesthetized with I.V. sodium pentobarbital to prevent shivering. Following tracheal intubation, the dog was hyperventilated with pure oxygen. When 29°C rectal temperature was obtained, the dog was removed from the bath; a further drop of 3 to 6 degrees of temperature occurred. Using aseptic technic a right anterolateral thorocotomy incision was made through the 5th intercostal space. After entering the right thorax the azygos vein was ligated, the pericardial sac opened widely and both cavae were occluded. Fifteen seconds later the ascending aorta was occluded with a non-crushing clamp about one inch distal to the coronary ostia. A control series of dogs received no medications; the experimental group was treated as follows: Some received prostigmine intravenously before occlusion of circulation. In others, either prostigmine or acetylcholine was injected into the root of the aorta until the heart rate was reduced to 10 to 25 beats per minute. At this point injection of prostigmine was stopped; however, since its action is so brief, acetylcholine required a slow continuous perfusion to maintain the slow heart rate. This method of administration into the clamped aorta is termed coronary perfusion. To produce cardiac slowing, the range of dosage for prostigmine proved to be one to 2 ml of 1-4000 solution. For acetylcholine from one to 3 mg

---

* Aided in part by a grant from the Faculty Council on Research of University of Colorado.

TABLE I. Summary of Data.

	No. dogs	Ventricular fibrillation	Resuscitation
Ventriculotomy as fibrillatory stimulus			
Control	23	23	0 of 23 attempts
Prostigmine in open circulation	15	7	5 of 5 "
Prostigmine by coronary perfusion	16	0	0 "
Acetylcholine	3	0	0 "
Catheterization of coronary sinus as fibrillatory stimulus			
			(Prostigmine given after fibrillation occurred)
Control	10	10	10 of 10 attempts
Prostigmine coronary perfusion	10	0	0 "

were required. A 5 cm incision was made in the right or left ventricle; occasionally a ventricular septal defect one cm in diameter was created. The septal defect was closed with interrupted sutures and after filling the heart with saline, the ventricular wall was also sutured. This accomplished, the aortic clamp was removed followed by simultaneous release of both cavae. The total occlusion time varied from 6 to 14 minutes, the average being 8 minutes.

Results. Right ventriculotomy is a fibrillatory stimulus in the cold dog. Our control series of 23 dogs all incurred ventricular fibrillation during ventriculotomy. Many succumbed to fibrillation with the initial needle-prick. Resuscitation by massage and electric shock was uniformly unsuccessful. Potassium chloride was not used(4). When prostigmine (.05 ml of 1-4000 solution per kg body weight) was administered in the general circulation before inflow occlusion to the heart, the incidence of fibrillation was reduced to about 50%. Table I shows that 8 of 15 dogs had no fibrillation. The remaining 7 suffered ventricular fibrillation. Resuscitation was successful in each of the 5 animals in which it was attempted. In the hope of a more direct administration of prostigmine to the heart yielding more consistent results, this agent was administered in another series of 16 animals by coronary perfusion. In this group ventriculotomy was performed without the occurrence of ventricular fibrillation. Eight of these dogs also had creation of a small ventricular septal defect with closure.

As an incidental finding to other work it was found that catheterization of the coronary sinus in the cold dog was attended by a high incidence of ventricular fibrillation. This maneuver was also used as a fibrillatory stimulus. Table I shows that 10 of 11 dogs subjected to this procedure incurred ventricular fibrillation. However, when prostigmine was given by coronary perfusion prior to catheterization, no ventricular fibrillation occurred in a group of 10 animals. It was found that when ventricular fibrillation occurred in the unprotected animals resuscitation was uniformly unsuccessful. After failure of the attempted resuscitation prostigmine was given by coronary perfusion with massage. Electric shock was then again instituted with 100% success. Seven of these dogs reverted directly from ventricular fibrillation to normal sinus rhythm following electric shock; the other 3 required massage. Since it seemed likely that the antifibrillatory effect of prostigmine was due to an accumulation of acetylcholine, this agent was administered by coronary perfusion in 3 animals. Table I indicates that ventriculotomy was successfully performed in all 3 of these animals without the occurrence of ventricular fibrillation.

Summary. Prostigmine is shown to have an antifibrillatory effect in the hypothermic dog. This agent is more effective when given by coronary perfusion than when given intravenously. When ventricular fibrillation occurs in the unprotected animal, coronary perfusion of prostigmine will allow conversion to a normal rhythm by massage and electric shock. It is felt that the antifibrillatory effect of prostigmine is probably due to an accumulation of acetylcholine.

---

1. Bigelow, W. K., Lindsay, J. C., and Greenwood, W. F., *Ann. Surg.*, 1947, v132, 849.

2. Rosenbain, F. R., and Penrod, K. E., *Am. J. Physiol.*, 1951, v166, 55.

3. Swan, H., Zeavin, I., Holmes, J. H., and Montgomery, V., *Ann. Surg.*, 1953, v138, 360.

4. Swan, H., Zeavin, I., Blount, S. G., and Virtue, R. W., *J.A.M.A.*, 1953, v153, 1081.

Received March 15, 1954. P.S.E.B.M., 1954, v85.

Chemical Studies in Experimental Scorbutus in Cortisone-Treated Guinea Pigs.* (20964)

CHARLOTTE L. MADDOCK. (Introduced by S. B. Wolbach.)

From the Division of Nutritional Research, Division of Laboratories and Research, The Children's Hospital, Boston, Mass.

In a previous communication the evidence that cortisone had no effect upon the sequence of events in both the production of and early repair from experimental scurvy in guinea pigs, using gross and histological changes as criteria, was documented(1). It has been suggested that depolymerization of ground substance, one of the components of intercellular substances, may occur in scurvy(2), and that the elevation of serum glycoprotein values in the scorbutic guinea pig, a fact established by the work of Pirani and Catchpole(3) may be a direct result of this depolymerization mechanism. If this is so, the action of cortisone upon scurvy might very well be reflected by alterations in these heightened values. If no effect is demonstrated, this would be compatible with the histological findings cited above. With these ideas in mind this study was initiated. It reports the serum mucoprotein values, as well as serum cholesterol and lecithin levels of scorbutic guinea pigs treated with cortisone.

Experimental. Forty-two guinea pigs were employed in this study, divided as indicated in Table I. In the scurvy plus "prophylactic" cortisone, cortisone was begun at the same time as the guinea pigs were placed on the scorbutic diet. Cortisone was given intraperitoneally at the following levels; to one animal 10 mg cortisone per kg of body weight, to two others 5 mg cortisone per kg of body weight. In the scurvy plus "curative" cortisone group, the guinea pigs were allowed to progress to the symptom stage before cortisone was given. Again this was administered intraperitoneally at the following levels—one at 10, two at 5, and one at 2.5 mg cortisone per kg of body weight. Two repair periods were selected in the subsequent categories, 72 and 96 hours respectively, during which time 50 mg of ascorbic acid per day was given by mouth to every animal. Guinea pigs were studied during these periods with and without cortisone administered intraperitoneally at the following levels, 2.5 and 5.0 mg cortisone per kg of body weight. In the prophylactic and curative cortisone groups the animals ranged in weight initially from 120 to 201 g, while in the repair group they were somewhat heavier, weighing between 248 and 300 g. Five additional untreated scorbutic animals, averaging 400 g in weight were used to determine mucoprotein tyrosine values. In both untreated scurvy and in control groups, intermediate weights were encountered. The diet used for produc-

TABLE I.
Mucoprotein Tyrosine and Glucosamine in Scorbutic Guinea Pigs with and without Cortisone.

			Serum	
	No. of animals	Avg duration of exp., days	Mucoprotein, tyrosine, mg % ± S.D.	Glucosamine, mg % ± S.D.
Scurvy	13	23.8	9.47 ± .088	93.25(8)* ± 1.177
" + "prophylactic" cortisone	3	28.3	9.96 ± .264	95.76 ± 6.474
" + "curative" "	4	25.8	9.70 ± .260	86.20(3)* ± .5102
Scurvy + repair—72 hr	2	26.0	9.55 ± .177	94.60 ± 1.929
idem —96 "	4	24.0	9.30 ± .210	101.60 ± .975
Scurvy + cortisone + repair—72 hr	2	26.0	8.50 ± .638	102.75 ± 1.241
idem —96 "	4	24.0	7.96 ± .167	97.63 ± 2.355
Controls	6	27.4	5.17 ± .190	74.05 ± 2.780

* No. in parentheses indicate number of animals used to determine avg for value in question.

---

* Aided by grants from the Williams-Waterman Fund for the Combat of Dietary Diseases.

TABLE II. Cholesterol and Lecithin in Scorbutic Guinea Pigs with and without Cortisone.

	No. of animals	Avg duration of exp., days	Serum		Ratio chol./leci.
			Cholesterol, mg % ± S.D.	Lecithin, mg % ± S.D.	
Scurvy	11	23.3	92.18 ± 2.71	168.02(8)* ± 9.87	.548
" + "prophylactic" cortisone	2	26.5	99.00 ± 10.28	122.00(3) ± 14.45	.812
" + "curative" "	6	24.2	117.45 ± 6.33	111.50(2) ± 3.18	1.052
Scurvy + repair—72 hr	3	26.7	109.46 ± 11.04	165.50(2) ± 22.97	.662
idem —96 "	4	24.0	188.75 ± 4.98	203.30 ± 5.60	.914
Scurvy + cortisone + repair—72 hr	5	27.4	127.46 ± 8.97	172.00(2) ± 9.22	.742
idem —96 "	4	24.0	194.75 ± 13.95	194.25 ± 10.60	1.002
Controls	23	—	52.20 ± 0.51	62.46 ± .75	.835

* No. in parentheses indicate number of animals used to determine avg for value in question.

ing the deficiency was ground Purina rat chow (checkers) heated overnight at 100°C, with 4% dried yeast added, and then mixed with sufficient water to moisten and thus make it more acceptable to the guinea pigs. One cc of cod liver oil was given twice weekly. This diet, supplemented with ascorbic acid (50 mg by dropper daily) was adequate for maintenance of good health in the control animals. The guinea pigs were kept in individual wire bottom cages, allowed food and water *ad libitum* and weighed daily. The duration of the experiment varied between 20 and 30 days, being determined in the "cortisone prophylactic" and "cortisone curative" group by the condition of the animals which were killed in the very late stages of scurvy. Scurvy controls and diet controls were killed at corresponding times so as to furnish adequate material for comparative tests. Bleeding was done by cardiac puncture under light ether anesthesia. Bloods were analyzed for total cholesterol by the Liebermann-Burchard method, for lipid-P, by the method of Fiske and SubbaRow, for mucoprotein tyrosine by Winzler's method(4),[†] and for serum glucosamine by a modification of Boas(5) of Elson and Morgan's method(6).

Results. The results are presented in tabular form. From Table I, one may deduce that cortisone, administered either "prophylactically" or "curatively" has no influence upon the level of mucoprotein tyrosine values in serum from scorbutic guinea pigs. Cortisone, administered together with ascorbic acid, appears to hasten the return toward normal values as compared to the results seen in guinea pigs recovering from scurvy with the aid of ascorbic acid alone. The glucosamine values, always considered less reliable because of the inherent difficulties in the procedure, show no downward trend in repair by cortisone and ascorbic acid.

Cholesterol and lecithin values, elevated in scurvy, are even higher when cortisone is administered to scorbutic animals (Table II). In early repair, also, the values are higher and rise with duration of repair, at least up to 96 hours, the longest time span studied. Cortisone thus further elevated the high values of the two lipid components studied in early repair.

Discussion. The results obtained were probably to be anticipated. Cortisone given to scorbutic animals does not in any way influence the course of the deficiency, the histology, or that part of the chemical picture that reflects the deficiency syndrome. In repair, mucoprotein tyrosine values tend to decline, a process that appears to be accelerated by the simultaneous administration of cortisone. This phenomenon is not reflected in the glucosamine values. We have no explanation for this except to say that there is no reason to expect an exact correspondence between these two sets of values, since the procedures employ different serum protein moieties. More reliance should be placed on the mucoprotein tyrosine results because of the greater accuracy and reproducibility of the method.

The higher cholesterol and lecithin values in cortisone "modified" scurvy may merely reflect a summation of the scurvy and the cortisone effect(7,8) on these particular lipid components of blood serum.

[†] A slight modification in procedure, *viz,* dilution of the blood serum with physiological saline, 1:1, before addition of the $HClO_4$ precipitating reagent, was used.

Summary. 1. Sera of guinea pigs suffering from uncomplicated scurvy and sera of scorbutic guinea pigs where cortisone was administered, do not differ from one another as regards the following values: mucoprotein determined as a) tyrosine; b) glucosamine. 2. Sera of scorbutic guinea pigs recovering from the deficiency through the administration of ascorbic acid approach normal mucoprotein tyrosine values more rapidly when cortisone is administered concurrently with ascorbic acid. 3. The administration of cortisone elevates the already high cholesterol and lecithin values in scorbutic guinea pigs.

---

1. Wolbach, S. B., and Maddock, C. L., *Arch. Path.*, 1952, v53, 54.
2. Gersh, I., and Catchpole, H. R., *Am. J. Anat.*, 1950, v85, 457.
3. Pirani, C. L., and Catchpole, H. R., *Arch Path.*, 1951, v51, 597.
4. Winzler, R. S., Devor, A. W., Mehl, J. W., and Smyth, I. M., *J. Clin. Invest.*, 1948, v27, 609.
5. Boas, Norman F., Personal communication to Paul R. Patterson.
6. Elson, L. A., and Morgan, W. T., *Biochem. J.*, 1933, v27, 1824.
7. Adlersberg, D., Schaefer, L. E., and Dritch, R., *J. Clin. Invest.*, 1950, v29, 795.
8. ———, PROC. SOC. EXP. BIOL. AND MED., 1950, v74, 877.

Received March 15, 1954. P.S.E.B.M., 1954, v85.

Effect of Cortisone upon Local Capillary Permeability. (20965)

VIRGIL H. MOON AND MARIA A. TERSHAKOVEC.

From the Medical Research Unit, University of Miami and the Veterans Administration Hospital, Coral Gables, Fla.

Among the important effects of the cortical steroids is their influence upon endothelium. Kendall(1) gave as their primary function, the control of permeability of cells, and of the transfer of inorganic ions and water between the cells and the extracellular space. The control of permeability applies to endothelium as well as to other cells. Physiologists (Best and Taylor(2)) agree that one property of cortisone is to maintain the impermeability of capillary endothelium. Ebert(3) and Lurie (4) reported that cortisone had this effect in reactions to injury; Balourdas and Chambers (5) found that cortisone caused reduction of capillary leakage in the exteriorized mesoappendix of rats. Whitelaw(6) used ACTH by injection in the treatment of burned patients and reported a definite decrease in the oozing from burned surfaces. Other such observations might be cited.

A simple experiment was devised to produce additional evidence upon the effects of cortisone upon capillary permeability. It is well known that trypan blue will cause staining in areas of increased permeability seen in inflamed or injured tissues. The test consists of injecting a solution of trypan blue intravenously, then applying to the skin the agent to be tested. The prompt development of a blue zone about the test point indicates that the capillaries there are abnormally permeable. We(7) have shown that extracts of normal tissues and various substances containing products of protein cleavage, will cause a local increase in permeability if injected into the skin.

Methods. A weighed amount of tissue was suspended finely in 4 volumes of 0.9% NaCl solution in a Waring Blendor. This was centrifuged, the supernatant fluid was adjusted to pH 7 and sterilized by filtration (Seitz). Solutions of substances containing products of protein digestion were sterilized in the same manner. These included Neo-peptone, Bactoprotone (each by Difco), Witte's peptone and a peptic digest of fibrin made in our laboratory. A series of dilutions was tested to determine what amounts would produce a positive trypan blue reaction. Such amounts of each extract and solution were tested in dupli-

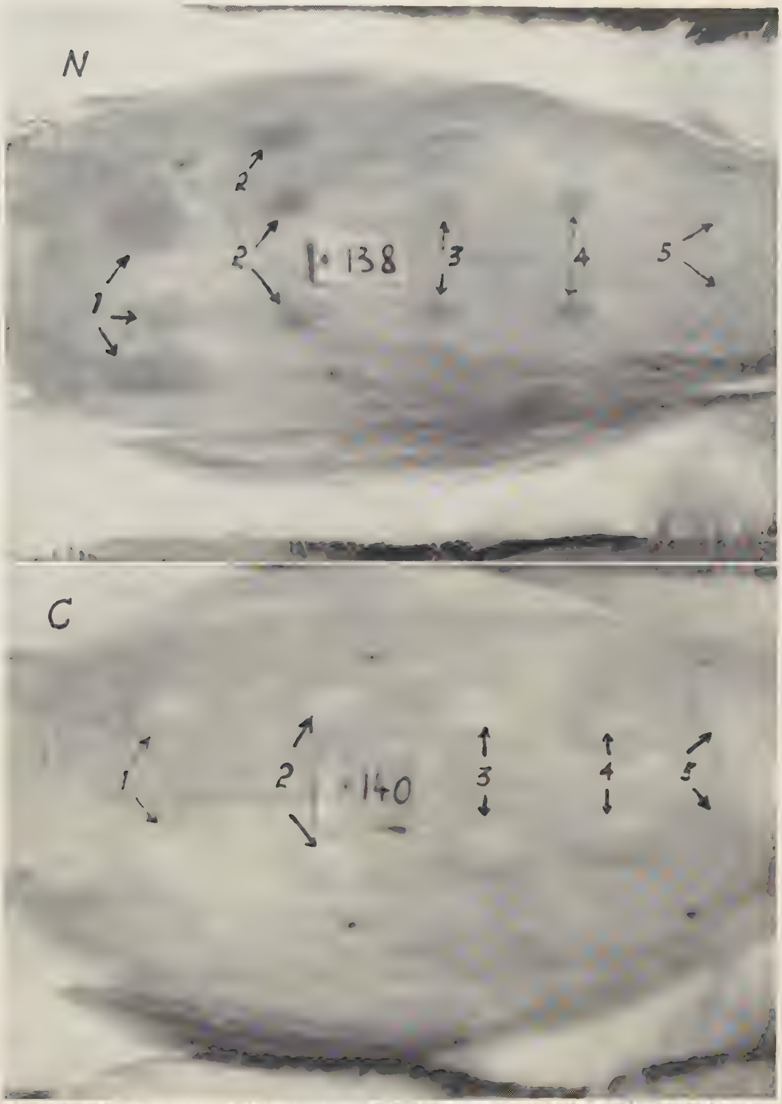


FIG. 1. Photograph of permeability tests. N indicates normal (#138); C indicates cortisone treated animal (#140). 1: histamine, 0.01%; 2: fibrin, peptic digest, 0.05%; 3: Neopeptone, 0.05%; 4: Bacto-protone, 0.05%; 5: NaCl solution, 0.9%, control.

cate on the same animal in each experiment. A 0.01% solution of histamine was used as a positive control, and a 0.9% solution of NaCl as a negative control. Healthy male New Zealand white rabbits were used. The fur over the abdomen was sheared closely with clippers, using care not to injure the skin. Then a freshly made 1% solution of trypan blue was injected intravenously, 1.0 ml per kg. An injection of 0.1 ml of the solution to be tested was made into the skin with a 26-gauge needle. Rabbits were used in pairs, one having received intramuscular injections of cortisone 18 hours and one hour before the injections; each dose contained 5 mg/kg of cortisone. The same agents in identical doses were injected into a control animal which had received no cortisone (Fig. 1). Very strong reactions were recorded as 3+, strong 2+, moderate 1+, doubtful ±, and negative —. The findings are given below in tabular form.

Results. It is observed that the results

TABLE I. Digests of Protein.

Substance	Dilution		Tests	3+	2+	1+	±	—
Neo-peptone	.0005	N*	12	3	2	4	1	2
		C*	14	0	0	0	0	14
Bacto-protone	.0005	N	14	6	8	0	0	0
		C	14	0	0	0	3	11
Fibrin digest	.0005	N	14	6	4	4	0	0
		C	14	0	0	1	2	11
Witte's peptone	.0005	N	6	6	0	0	0	0
		C	6	0	0	2	0	4
Histamine	.0001	N	10	5	3	2	0	0
		C	10	0	0	0	2	8

* N indicates normal; C indicates cortisone treated animals.

varied somewhat even when the agents, the dilutions and the dosages were identical. A minor factor in this may have been a difference in the responses of individual animals. But it is noted that the variations were much greater in the reactions to extracts of tissues than to the products of protein digestion. We regard it as significant that the reactions to histamine were quite uniform. Now histamine is a chemical entity whose composition is known. Some have assumed that extracts of tissues produce increased capillary permeability because they contain a chemical entity—"leukotoxine". If this were true, then the extracts should evoke a more uniform response as do histamine and certain digests of protein.

The tabulated results indicate that cortisone, given intramuscularly prior to experimentation, tends to maintain the impermeability of endothelium against various agents which produce capillary permeability in untreated animals. This finding does not coincide with that of Shleser and Freed(8), who reported that Compound E (cortisone) when injected intradermally together with a 2% solution of Bacto-peptone, did not prevent capillary permeability. Menkin(9) reported that cortisone acetate alone, injected intradermally, induced a rapid and constant increase in capillary permeability as shown by the trypan blue test. The dose injected into the skin may differ from the effect of the same hormone introduced systemically.

Specimens of skin in test areas were sectioned and stained routinely for histologic examination. Some were taken one hour after the test, others after 2.5 hours. The lesions from the untreated animals regularly showed acute edema and beginning leukocytic infiltration after one hour; these features were more advanced 2.5 hours after the injections. The acute inflammation was most pronounced in the lesions produced by Bacto-protone, peptic digest, and by extracts of spleen and of lung. Both edema and leukocytic infiltration were of lesser degree in the cortisone-treated rabbits. This finding confirms our previous report(10) and the observations of others that cortisone inhibits the development of acute inflammation. One observation was of special interest: in the untreated animals, the leukocytes had migrated freely from the minute vessels into the tissue spaces. In contrast, in the cortisone-treated animals, the leukocytes came to the area but remained mostly within the lumina of the capillaries and

TABLE II. Extracts of Tissue.

Substance	Dilution		Tests	3+	2+	1+	±	—
Kidney	1/8	N*	9	8	1	0	0	0
		C*	6	0	2	2	2	0
"	1/16	N	12	8	4	0	0	0
		C	8	0	2	4	2	0
"	1/64	N	5	2	2	1	0	0
		C	5	0	0	0	0	5
"	1/128	N	4	2	0	2	0	0
		C	4	0	0	0	0	4
Lung	1/8	N	4	4	0	0	0	0
		C	4	0	0	2	1	1
"	1/16	N	6	2	2	2	0	0
		C	4	0	0	0	2	2
Spleen	1/2	N	6	6	0	0	0	0
		C	4	0	0	2	2	0
"	1/4	N	6	2	3	1	0	0
		C	4	0	0	0	2	2

* N indicates normal; C indicates cortisone treated animals.

venules; relatively few had migrated into the tissue spaces. This observation suggests that leukocytes migrate more readily through the walls of capillaries whose permeability has been increased. The capacity of cortisone to maintain endothelial impermeability is probably an important factor in its retarding effect upon acute inflammation.

Summary. 1. Various products of protein digestion and extracts of normal tissues caused increased capillary permeability when injected intradermally. This effect was reduced greatly by pretreatment with cortisone. 2. Local edema and leukocytic infiltration developed at the site of the injections. These effects were more acute in the untreated animals. There was evidence that cortisone inhibited the emigration of leukocytes from within the minute vessels.

---

1. Kendall, E. C., *The Adrenal Cortex. Arch Path.*, 1941, v32, 474.

2. Best, C. H., and Taylor, N. B., *The Physiologic Basis for Medical Practice,* Baltimore, Williams and Wilkins Co., ed. 5, 1950, p313.

3. Ebert, R. H., *J. Clin. Invest.*, 1951, v30, 636.

4. Lurie, M. B., Zappasodi, P., Dannenberg, A. M., Jr., and Lynch, E. C., Abstr., 47th Ann. Meet., *Nat. Tuberc. Assn.*, May 14, 1951, p4-5.

5. Balourdas, T., and Chambers, R., *Fed. Proc.*, 1952, v11, 8.

6. Whitelaw, M. J., Proc. 2nd Clin. ACTH Conf., Philadelphia, Blakiston, 1951, p310.

7. Moon, V. H., and Tershakovec, Geo. A., *A. M. A. Arch. Path.*, 1953, v55, 384.

8. Shleser, I. H., and Freed, S. C., *Am. J. Physiol.*, 1942, v137, 426.

9. Menkin, V., *Am. J. Physiol.*, 1951, v165, 294.

10. Moon, V. H., and Tershakovec, Geo. A., PROC. SOC. EXP. BIOL. AND MED., 1952, v79, 63.

Received March 22, 1954. P.S.E.B.M., 1954, v85.

Curare-Like Activity of Some Bis-Fluorenyl-Bis-Quaternary Ammonium Compounds. (20966)

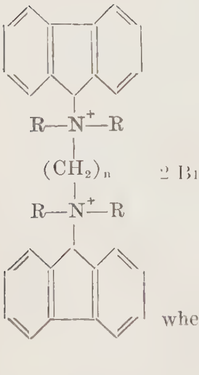
FRANK J. MACRI. (Introduced by T. Koppanyi.)

From the Research Laboratories, Irwin, Neisler & Co., Decatur, Ill.

Bovet(1) has proposed a very interesting physical distinction between those agents which induce myoneural junction blockade by either depolarization or stabilization of the end-plate. He proposes the term "leptocurare" for agents which induce blockade by depolarization and for which the molecular configuration usually is long and thin, *i.e.*, decamethonium. "Pachycurare" is proposed for those agents which induce blockade by stabilization of the membrane, such as d-tubocurarine, and for which the molecular configuration is quite bulky in width. In the reports of Barlow and Ing(2,3), Paton and Zaimis(4,5) and in the excellent reviews of Paton(6) and Riker(7), it has been observed that maximum curariform activity, with bisquaternaries, is obtained when the distance between quaternary nitrogen atoms is approximately 15 Å, irrespective of the type of blockade instituted.

This is a report on the neuromuscular blocking properties of a new series of compounds prepared by Gray *et al.*(8), in which the nitrogen atoms are joined by a polymethylene chain and in which one of the groups on each nitrogen atom is a fluorenyl group. The compounds are of interest in that the large terminal groups modify the distance between nitrogen atoms which have been reported necessary for optimal activity. A similar modification of activity by terminal groupings was reported by Wien *et al.*(9) for the ganglionic blocking agents in the methonium series.

Methods. 1) *Mice.* The inclined screen procedure of Thompson(10), as modified by Hoppe(11), was used for the quantitative determination of the potency of these compounds. Concentrations were varied by equal increments so that at least 3 reactive groups were obtained between the ED_0 and the



where $R = CH_3$, $n = 6$ (IN-117)
$R = C_2H_5$, $n = 6$ (IN-137)
$R = C_2H_5$, $n = 10$ (IN-138)

ED_{100}. The mice were run in groups of 10 and the concentration adjusted so that 0.01 ml of solution was administered per 2 g of body weight. The subcutaneous absorption of these compounds was found to be very slow, requiring from one-half to 3 hours for the onset of paralysis; therefore, those mice which became paralyzed and slid off the screen within 3 hours were considered positive reactors. Twenty minutes was sufficient time to obtain maximum reactors by the intravenous route of administration. The ED_{50} and LD_{50} were calculated by the method of Behrens (12). 2) *Rabbits.* Intravenous doses of increasing equal increments were administered to rabbits in groups of 5. Concentrations of solutions were adjusted so that 0.1 cc was administered per kg of body weight. A reactor was considered positive when a "head drop" was obtained to the extent that the head rested on the table top and could not be raised, even by prodding. The dose producing "head drop" in 50% of the animals (HD_{50}) was calculated by the method of Behrens(12). 3) *Dog.* Myoneural junction transmission. Dogs were anesthetized with pentobarbital sodium (35 mg/kg) intravenously. The tendon of Achilles was sectioned just proximal to the calcaneous by cautery, to avoid bleeding. A heavy thread was tied around the tendon, which was then led over a pulley and to which was attached a 200 or 300 g weight. A second thread was tied to the thread of the tendon in such a manner that through a system of pulleys a lever reflected upwards for every contraction of the gastrocnemius muscle. The peripheral end of the sectioned tibial nerve was stimulated electrically with currents just above threshold values at a rate of 15/min. A half-hour period of stimulation was allowed for stabilization at which time the current intensity was redetermined to be certain that it was still close to the threshold value. If the contractions were of uniform height during another 15-minute stabilization period, the determination of the myoneural junction blocking dose of these compounds was begun. Injections were made in the femoral vein at intervals of 3 hours. More rapid injections led at times to a lessening of the dose which would completely block the gastrocnemius twitch. At least 10 determinations were made of the dose which was just below and just above the dose which gave a complete inhibition of the twitch. By this process of "closing-in", reproducibility within 5% was obtained.

Results. Mice. The group of bis-fluorene compounds tested in mice demonstrated activity less than that which was obtained by d-tubocurarine, whether administration was by the intravenous or subcutaneous route. The intravenous ED_{50} values (determined by the inclined screen procedure) of IN-117, IN-137, and IN-138 were 4.3%, 7.5% and 7.7%, respectively, as active as d-tubocurarine. The ratios ED_{50}/LD_{50} of these agents in the above order were 0.72, 0.62 and 0.93. From these ratios it can be seen that there was a very slight spread between the ED_{50} and LD_{50} of IN-138, but an appreciable spread was obtained with IN-117 and IN-137.

TABLE I. Comparative Pharmacology of IN-117, IN-137, IN-138 and d-Tubocurarine.

			d-Tubo	IN-117	IN-137	IN-138
ED_{50}	Mice	I.V.	.054*	1.26	.72	.70
LD_{50}	"	"	.14	1.76	1.16	.75
LD_{100}	"	"	.21	2.02	1.6	
ED_{50}	"	S.C.	.275	238	14.7	
LD_{50}	"	"	.525	240	19.95	
LD_{100}	"	"	.70	400	40.0	
ED_{50}	"	Oral		280		
LD_{50}	"	"		280		
LD_{100}	"	"		400		
HD_{50}	Rabbit	I.V.	.161	0.08	.09	.205
LD_{50}	"	"	.223	0.11	.11	.220
LD_{100}	"	"		0.12	.12	.25
Myoneural junct. block (dog)			.24	0.21	.21	.66

* Doses in mg/kg.

The subcutaneous ED_{50} and LD_{50} of IN-117 and IN-137 differed markedly between the 2 compounds. The ED_{50} of IN-117 was found to be 149 and the LD_{50} 137 times the dose required to obtain these responses by the intravenous routes. For IN-137, the ED_{50} S.C./ED_{50} I.V. and LD_{50} S.C./LD_{50} I.V. were found to be 20 and 17, respectively. It is probable that a slow rate of absorption can account for the very high values obtained with IN-117. That IN-117 has such a markedly slow rate of absorption is interesting since it differs from IN-137 only by the substitution of methyl for ethyl groups on the nitrogen atoms. Since this factor of absorption can occur, extreme caution should be practiced in the quantitative evaluation of "structure-activity" data which are obtained by routes of administration other than the intravenous.

Rabbits. IN-138 responses were similar to those of d-tubocurarine in the rabbit. Doses 3 times greater than the mouse I.V. ED_{50} were required to produce the HD_{50}. IN-117 and IN-137, however, produced the HD_{50} with doses which were 0.06 and 0.12 times their respective I.V. mouse ED_{50}. Onset of action was apparent within one minute after full curarizing doses were administered. In subcurarizing doses the onset was delayed from 2 to 5 minutes. Recovery was noted within 10 minutes in rabbits which lived after a "head drop". The LD_{50} values obtained paralleled quite closely those values obtained for the HD_{50}.

Dog. IN-117, IN-137 and d-tubocurarine produced blockade of the myoneural junction in almost equivalent doses. However, IN-138, the decamethylene analog, could only induce complete blockade, with doses 3 times those of the former agents. Complete recovery of the myoneural junction blockade in dogs after the administration of "effective" doses of IN-117 was between 15-30 minutes. It was found that C-10 (0.2 mg/kg) immediately antagonized the complete myoneural junction blockade induced by IN-117. Neostigmine (0.05 mg/kg) antagonized the blockade for about one minute; the blockade then being again reinstituted. Further administration of neostigmine had no further effect on the blockade.

Other actions. IN-117 was infused into an anesthetized artificially respired dog at various rates over a 6-hour period. After a total of 91.2 mg/kg had been administered, it was found that the H.R. had decreased from 162 b.p.m. to 144 b.p.m. The blood pressure had decreased from 115 mm Hg to 80 mm Hg.

A large dose of epinephrine (0.05 cc of a 1:1000 solution) was administered at the completion of the experiment to determine cardiac irritability. The heart did not go into fibrillation. IN-117 did not cause inhibition of the response of stimulation of the peripheral end of the vagus as reported for curare by Claude Bernard. In atropinized, anesthetized dogs, it was found that the nicotinic blood pressure response of Ach was potentiated by IN-117, the degree of potentiation being greater with increasing doses of IN-117. In mice, the "essential elimination" rate is 0.32 mg/kg/min.

Discussion. A new group of myoneural junction blocking agents is reported which demonstrates myoneural junction blockade in the dog and rabbit when the distance between nitrogen centers is approximately 9 Å. IN-137, in which the nitrogen atoms are separated by a 6-carbon chain and in which the nitrogen atoms contain 2 ethyl as well as a fluorenyl substituent demonstrated the greatest activity in all the animals tested. When the ethyl groups of the quaternized nitrogen are replaced by methyl groups (IN-117) the activity is markedly diminished in the mouse but is retained in the dog and rabbit. The replacement with the methyl groups of IN-117 also demonstrates what very probably is a very marked slowing of the rate of subcutaneous absorption. Doses in the order of 150 times the effective intravenous dose are required to produce 50% responses in mice, whereas with the ethyl analog a dose increment of approximately 20 times the effective intravenous dose is required to produce the same response.

Increasing the chain length of IN-137 from 6 carbons to 10 carbons (IN-138) induces no appreciable change in activity as measured in mice. However, activity is reduced approximately 66% from that obtained with IN-137 when rabbit head drop and dog myoneural junction blocking doses are compared.

Conclusions. 1. The curariform activity of a new series of bis-quaternary ammonium compounds is reported. 2. Moderate increase of activity is obtained by shortening the distance between nitrogen atoms from approximately 15 Å to 9 Å. 3. Replacement of N-ethyl groups with N-methyl in the 6-carbon chain analog decreases both the activity and the subcutaneous absorption in mice, but has no effect on the activity as measured in dogs and rabbits.

The technical assistance of Mr. Cris Luna is gratefully acknowledged.

1. Bovet, D., *Ann. N. Y. Acad. Sci.*, 1951, v54, 407.

2. Barlow, R. B., and Ing, H. R., *Nature*, 1948, v161, 718.

3. ———, *Brit. J. Pharmacol.*, 1948a, v3, 298.

4. Paton, W. D. M., and Zaimis, E. J., *Nature*, 1948, v161, 718.

5. ———, *ibid.*, 1948a, v162, 810.

6. Paton, W. D. M., *J. Pharm. Pharmacol.*, 1949, v1, 273.

7. Riker, W. F., Jr., *Am. J. Med.*, 1953, v15, 231.

8. Gray, A. P., Spinner, E. E., Schlieper, D. C., and Cavallito, C. J., in press.

9. Wien, R., Mason, D. F. J., Edge, N. D., and Langston, G. T., *Brit. J. Pharmacol.*, 1952, v7, 534.

10. Thompson, R. E., *Endocrinology*, 1946, v39, 62.

11. Hoppe, J. O., 1950.

12. Behrens, B., and Kärber, G., *Arch. f. exp. Path. u Pharmakol.*, 1934, v177, 379.

Received March 22, 1954. P.S.E.B.M., 1954, v85.

Peptidases in Isolated Blastomeres of *Mytilus edulis.** (20967)

WILLIAM E. BERG. (Introduced by Richard M. Eakin.)

From the Department of Zoology, University of California, Berkeley, Calif.

Investigations of mosaic eggs of annelids and mollusks in attempts to characterize the various cytoplasmic regions in chemical terms have demonstrated regional differences in pH, rH, enzymes and oxidative metabolism, Spek(1), Ries(2), Berg and Kutsky(3). The following investigation was undertaken to see if regional differences of peptidases might occur in a mosaic egg.

Peptidases have been found in eggs and embryos of invertebrate animals, Holter(4), Linderstrøm-Lang and Holter(5), Holter, *et al.*(6). No correlation of enzymatic activity with cell determination has been shown in the regulative egg of the sea urchin, Holter and Lindahl(7). Alanylglycine peptidase has been found in the mosaic eggs of *Tubifex*, Holter *et al.*(6), and of *Chaetopterus*, Holter(4); however, the localization of the enzyme and correlation with morphological differentiation was not investigated. The eggs of *Mytilus edulis*, used in this study, undergo a mosaic development as shown by differentiation of isolated blastomeres, Rattenbury and Berg (8). The most obvious segregation of factors important to subsequent differentiation occurs at the first cleavage, and methods have now been developed for removal of the egg membranes and separation of the first cleavage blastomeres, Berg(9). Measurements of peptidases in first cleavage blastomeres, reported here, are in part the results of an investigation of the physiological and chemical properties of the isolated early cleavage blastomeres.

Methods. The gametes of *M. edulis* were obtained by allowing individual animals to spawn in dishes of sea water. Vitelline membranes were removed with a membrane lysin and the first cleavage blastomeres separated in calcium free sea water. Peptidases were determined by a modification of the titrimetric method of Linderstrøm-Lang and Holter(10). The segregated blastomeres were cytolyzed in 7.4 μl glycerol-phosphate solution. 7.4 μl of substrate (alanylglycine or leucylglycine) were added and the mixture incubated

---

* Aided in part by grants from the Committee on Growth acting for the American Cancer Society and from the University of California Board of Research.

TABLE I. Alanylglycine Peptidase Activity in Early Developmental Stages of *Mytilus edulis*.

Stage of development		Peptidase activity, μl 1/20 N HCl/embryo
Unfertilized egg		.34 ± .013
Fertilized ”		.36 ± .013
Blastula	6 hr	.36 ± .007
Gastrula	12 ”	.34 ± .017
Trochophore larva	24 ”	.30 ± .015
Veliger ”	48 ”	.12 ± .012

at 40°C for 18-20 hours. After stopping the reaction with 30 μl of 0.05 N alcoholic acid, 150 μl of acetone were added and the mixture was stirred. 150 μl of this were transferred to a cup shaped micro glass electrode and, using a Beckman pH meter, titrated to an end point with 0.05 N alcoholic acid from a micro burette. Electrometric titration was found to be more convenient and reliable than the colorimetric titration with naphthyl red as originally used by Linderstrøm-Lang and Holter. Since extremely small amounts of peptidase may be quantitatively measured by this method, relatively few blastomeres were needed for each determination. The majority of tests were carried out on 9 CD and 18 AB blastomeres which represent equivalent amounts of cytoplasm. To obtain accurate measurements of amounts of cytoplasm, the diameters of the two types of blastomeres were measured with an ocular micrometer. Peptidase activities were expressed as μl of acid used for titration. The experimental error in this technic is considerable; accordingly, sufficient determinations were made to permit a statistical evaluation of the results.

Results. Alanylglycine peptidase activity was found to be present in unfertilized eggs and all stages of early development of *Mytilus* (Table I). Ten or more measurements of peptidase were made on homogenates of the developmental stages. There is no significant variation of the total amount of the enzyme activity during cleavage and gastrulation; however a significant decrease occurs at the trochophore stage and the veliger contains only one-third the activity of early stages.

TABLE II. Alanylglycine Peptidase Activity in Isolated Blastomeres of *Mytilus edulis*

Blastomere	Titration value, μl 1/20 N HCl/cell	Avg vol of cells, 10^{-5}mm^3	Peptidase activity, 10^3 μl 1/20 N HCl/mm^3 cytoplasm
AB	.13 ± .01	4.1 ± .08	3.2 ± .31
CD	.24 ± .02	8.4 ± .13	2.9 ± .32

Localization of peptidases was investigated by measuring both alanylglycine and leucylglycine peptidases in isolated cleavage blastomeres. Table II summarizes the results of determinations of alanylglycine peptidase in AB and CD blastomeres. The results are averages of 9 experiments in each of which 4 to 6 separate determinations of peptidase were made on the isolated blastomeres. Analyzed statistically as a paired series, the activities of this peptidase are not significantly different in the 2 types of blastomeres.

Table III summarizes the measurements of leucylglycine peptidase in isolated blastomeres. Six hundred AB and 300 CD blastomeres were homogenized in 150 μl of extraction medium and 12 determinations of leucylglycine peptidase were carried out on the extract. The values for cell volumes from Table II were used for calculating relative enzyme activities. The activity of this enzyme was less than of alanylglycine which probably accounts for the greater variation of the measurements. Analyzed statistically as an unpaired series, there is no significant difference in activity of the enzyme in the blastomeres.

TABLE III. Leucylglycine Peptidase Activity in Isolated Blastomeres of *Mytilus edulis*.

Blastomere	Titration value, μl 1/20 N HCl/cell	Avg vol of cells, 10^{-5}mm^3	Peptidase activity, 10^2 μl 1/20 N HCl/mm^3 cytoplasm
AB	.021 ± .004	4.1 ± .08	5.1 ± .95
CD	.043 ± .005	8.4 ± .13	5.1 ± .66

It should be emphasized that these results are based on measurements of enzyme activity under optimal *in vitro* conditions and not the *in vivo* activity. As pointed out by previous investigators, enzymes in the living cell may be influenced by activators, inhibitors, conditions of pH, etc., and thus measurements of activity of an enzyme in homogenates may or may not reflect the actual activity in the cell.

Expressed as relative *in vitro* activities, the results may be compared with those obtained on other forms. The data of Table I indicate that during early development through gastrulation of *Mytilus,* the activity of alanylglycine peptidase remains constant. The uncleaved egg thus seems to be provided with sufficient amounts of this enzyme for subsequent development. These results are similar to those obtained by Holter and Lindahl(7) where the amount of alanylglycine peptidase remained constant to the pluteus stage of *Paracentrotus.* Linderstrøm-Lang and Holter(5) found a decrease in peptidase in later stages of development of *Urechis* which is similar to that which occurs in the veliger stage of *Mytilus.* This may be due to a decrease in growth rate with a lowering of synthetic metabolism in which peptidases are thought to play a role.

Holter and Lindahl(7) and Holter, *et al.* (11) found no correlation of peptidase activity with cell determination of the regulative egg of the sea urchin. Animal and vegetal regions of the embryo as well as isolated germ layers contained equal activities of peptidase. Likewise, according to the data in Tables I and II, in the mosaic egg of *Mytilus,* there is no correlation of peptidase activity with cell determination. Development of isolated blastomeres of *Mytilus* demonstrates a definite segregation of developmental potentialities at the first cleavage, however, these cannot be related to differences in peptidase activity. Ries(2) has shown by cytochemical technics a localization of the enzymes peroxidase and indophenol blue oxidase (presumably cytochrome oxidase) in some mosaic eggs; however, it would be desirable to measure these enzymes quantitatively. Cytochrome oxidase, as measured by a micro-spectrophotometric method has been found in both types of isolated blastomeres of *Mytilus*; however, the variability of the results prevented comparison of concentrations. As more micro methods are developed for measuring enzymatic activity it may be possible to demonstrate a localization of other enzymes in mosaic eggs. Also, the large scale segregation of isolated polar lobes from the eggs of *Mytilus,* Berg(12), brings the amount of specific cytoplasm available for quantitative biochemical studies into a range where ultra micro-chemical methods will not be needed.

Summary. Alanylglycine peptidase occurs in unfertilized eggs, cleavage and later developmental stages of *Mytilus edulis.* Peptidases, with alanylglycine and leucylglycine as substrates, were determined quantitatively in isolated first cleavage blastomeres. No significant differences in activities of these enzymes were found and it is concluded that the segregation of developmental factors at the first cleavage is not related to activities of these enzymes.

---

1. Spek, J., *Protoplasma,* 1934, 394.
2. Ries, E., *Pubbl. Staz. Zool. Napoli,* 1937, v16, 363.
3. Berg, W. E., and Kutsky, P., *Biol. Bull.,* 1951, v101, 47.
4. Holter, H., *J. Cell. Comp. Physiol.,* 1936, v8, 179.
5. Linderstrøm-Lang, K., and Holter, H., C. R. Carlsberg Lab., 1931, v19, 1.
6. Holter, H., Lehman, F. E., and Linderstrøm-Lang, K., *Z. f. Physiol. Chem.,* 1937, v250, 237.
7. Holter, H., and Lindahl, P. E., *C. R. Lab.,* Carlsberg, 1940, v23, 249.
8. Rattenbury, J., and Berg, W. E., to be published, 1954.
9. Berg, W. E., *Biol. Bull.,* 1950, v98, 128.
10. Linderstrøm-Lang, K., and Holter, H., *Z. f. Physiol. Chem.,* 1931, v201, 9.
11. Holter, H., Lanz, H., and Linderstrøm-Lang, K., *J. Cell. Comp. Physiol.,* 1938, v12, 119.
12. Berg, W. E., *Exp. Cell Res.,* 1954, v6, 202.

Received March 23, 1954. P.S.E.B.M., 1954, v85.

Serum Lipoproteins in Experimental Diabetes. I. Serum Lipoprotein Pattern of Normal and Depancreatized Dogs.* (20968)

ANGELO FASOLI,[†] EDWARD B. MAGID, MORTON D. GLASSMAN, AND PIERO P. FOÀ.
From the Department of Physiology and Pharmacology, Chicago Medical School, Chicago 12, Ill.

Studies of the past few years on serum lipoproteins suggest that the nature of the protein carrier and the type of linkage between protein and lipid are of fundamental importance in determining the metabolic fate of the lipid moiety. Experimental and clinical evidence further suggests that the *in vivo* transformation of serum lipoproteins usually proceeds from the larger molecules to the smaller ones, the former being the immediate result of fat absorption or mobilization, the latter being most probably related to lipid utilization in cell metabolism(1-6). Gofman and co-workers(7) reported that a strong statistical correlation exists between an increased concentration of certain lipoproteins (Sf_{10-20} and Sf_{20-100}) and clinical and experimental atherosclerosis suggesting an etiologic relationship. In view of the fact that atherosclerosis is more frequent in diabetic than in non-diabetic subjects, the lipoprotein pattern of diabetic patients has been analyzed in several laboratories and, although some results suggest an increase in the Sf_{10-20} and Sf_{20-100} fractions(8), others do not(9).

A study of serum lipoproteins in various types of experimental diabetes, under different conditions of treatment, seemed therefore of particular interest. In this paper, data will be presented on the lipoprotein patterns of normal and depancreatized dogs, with and without insulin treatment, as determined by zone-electrophoresis on filter paper.

Material and methods. Twenty normal dogs, kept on a constant diet of commercial dog food, were fasted about 18 hours. Blood samples were obtained, the serum separated by centrifugation and analyzed to establish normal lipoprotein pattern of the animal. Nine dogs were depancreatized and kept on a constant diet of raw horse meat, sucrose and raw pancreas in quantities sufficient to prevent fatty infiltration of the liver(10). After recovery from the operation, insulin requirement for complete control of glycosuria in 24-hour urine specimen was determined for each animal and varied between 10 and 40 units. Dose of insulin was then diminished or insulin withdrawn completely for several days, daily measurements of the 24-hour output of urine and glucose continued, and the serum lipoprotein pattern studied under various degrees of diabetic control. Forty lipoprotein determinations were made. Qualitative tests for ketone bodies in urine and blood were performed daily. Quantitative determinations of blood glucose and ketone bodies were made periodically and repeated every time lipoprotein analysis was performed. Fractionation of lipoproteins was performed as previously described(1,2) with an apparatus designed according to Durrum(11) and Flynn and de Mayo(12) with minor modifications. Strips of filter paper (Schleicher and Schull 598) 11 × 45 cm were used as support for electromigration and placed in the tank with the ends immersed in barbital buffer at pH 8.6 and μ 0.05. When the front of the "buffer" had climbed to within a few centimeters from the middle of the strip, serum (0.4 ml) was applied to the middle of the strip with a graduated pipette, as a transverse band as uniform as possible. After a few minutes to allow equilibration between serum and buffer, the circuit was closed, and a current of 140-160 volts and about 2 mA/strip, was allowed to flow from a rectifier. Electrophoresis was continued for 16 hours at room temperature. The strips were then dried in oven at 90°C for one half hour and cut longitudinally in 3 parts, 2, 2 and 5 cm wide respectively. The 2 narrow strips were used to identify the various lipoprotein fractions: one was treated with bromphenol blue to stain the proteins and the other with Sudan III to stain the lipids. The intensity of color was measured with the Beckman spectrophotometer by means of a

---

* This work was done under contract with Office of Naval Research.

[†] International Exchange Fellow: Present address—Clinica Medica, Università di Milano, Italy.

special attachment. The largest strip was then cut into sections corresponding to the position of the bands stained by Sudan III. Each section was extracted 3 times with boiling alcohol-ether mixture (3:1), extracts were pooled into a 10 ml glass-stoppered cylinder and, after cooling, the volume was brought to 10 ml with alcohol-ether mixture. A portion of each extract was analyzed for total cholesterol according to the methods of Bloor, Pelkan and Allen(13) adapted to spectrophotometry. Another portion was analyzed for lipid phosphorus according to the method of Youngburg and Youngburg as modified by Horwitt(14), after digestion with 10% perchloric acid. Portions of the serum itself were analyzed similarly. Total cholesterol and lipid phosphorus content of the various fractions were calculated as per cent of total amount recovered from the strip, and as per cent of total amount found in the serum. Recovery of the lipids from the strip was greater than 90%.

Results. Most of the lipids of dog serum are in complexes with high electrophoretic mobility. These constitute a group of fractions whose mobility ranges from that of α_2-globulins to albumin: boundaries between these fractions are often difficult to recognize and a broad band covering albumin-α-globulin area appears after staining with Sudan III. In a few normal sera there appears a distinct band between the α_1 and α_2-globulin. In addition, in some normal sera, lipids can be extracted from a zone immediately ahead of the albumin fraction, where, under certain conditions, significant amounts of lipoprotein migrate(15). The slow-migrating fractions, *i.e.*, the β-lipoproteins, and the fraction which does not migrate at all in filter paper electrophoresis and which will be referred to as the $\beta_2\gamma$-fraction, are present in low concentration in normal post-absorptive dog serum and account only for 15-20% of the total serum lipids (Fig. 1).

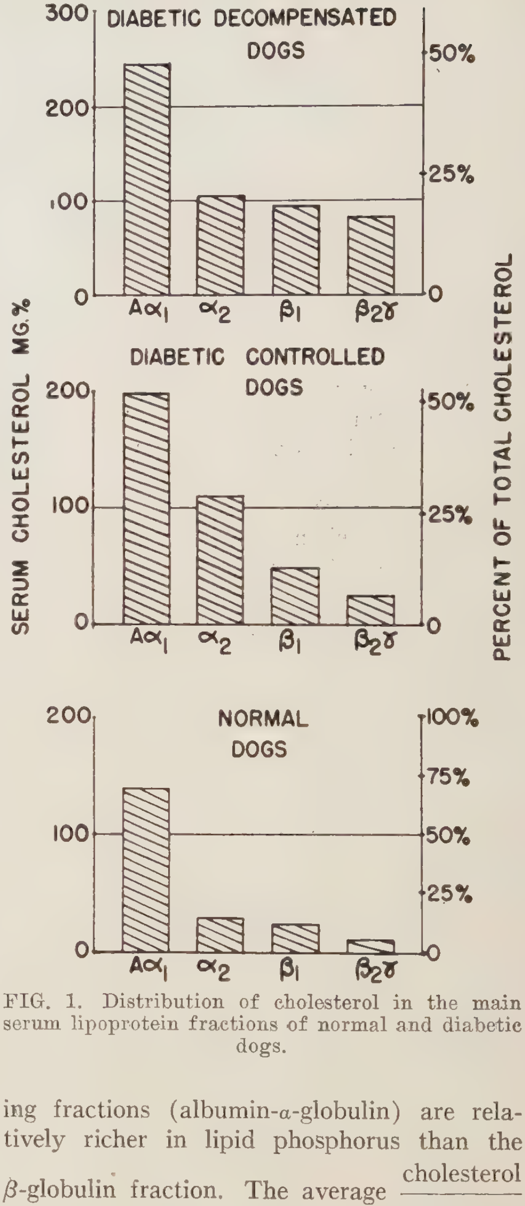


FIG. 1. Distribution of cholesterol in the main serum lipoprotein fractions of normal and diabetic dogs.

Fig. 2 reproduces protein and lipid pattern of normal dog serum as obtained through photometric evaluation of stained protein (bromphenol blue) and lipid (Sudan III) moieties. Lipid composition varies markedly from fraction to fraction. Thus the fast-moving fractions (albumin-α-globulin) are relatively richer in lipid phosphorus than the β-globulin fraction. The average $\frac{\text{cholesterol}}{\text{lipid P}}$ ratio is 18.3 for the albumin-α-globulin and 31.4 for the β-globulin group (Table I). This difference is statistically significant ($P = .03$). Comparison of the intensity of Sudan III stain of various fractions with cholesterol and lipid phosphorus distribution suggests that β fractions are richer in neutral fat than the fast-moving lipoproteins. This tendency of neutral fat to attach itself to the β lipoproteins was observed also after a fatty meal

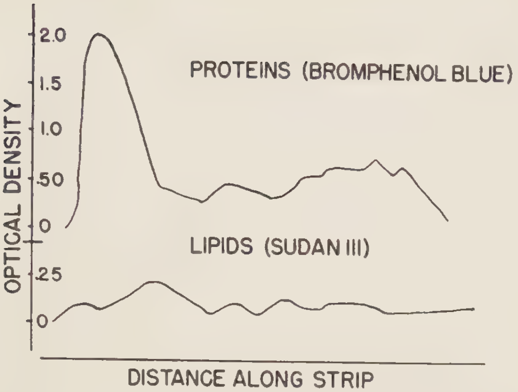


FIG. 2. Protein and lipid patterns of normal dog serum. Galvanometer readings. Instrument set at zero with unstained portion of strip.

when extra neutral fat appeared mostly in the $\beta_2\gamma$ fraction(15).

Depancreatized dogs under insulin treatment. In this group of animals, the level of serum cholesterol was consistently higher than in the control group. Total lipid phosphorus was also increased, but less than cholesterol. Relative distribution of lipids between the two main lipoprotein groups did not differ from normal serum, but often there was a change in the subfractions of the albumin-α-globulin group with a rise in the α_2-components, apparently at the expense of the faster migrating ones (Fig. 1). In this group of animals, the $\frac{\text{cholesterol}}{\text{lipid P}}$ ratio in whole serum and in albumin-α-globulin fraction was higher than in the normal animal. As in the normal animal, the value of the ratio was lower in the albumin-α-globulin than in the β-globulin fraction.

TABLE I. $\frac{\text{Cholesterol}}{\text{Lipid phosphorus}}$ Ratio in Alpha and Beta Lipoproteins of 15 Normal Dogs.

	Albumin-α-lipoprotein	β-lipoprotein
	14.9	27.8
	18.3	16.0
	17.8	26.6
	18.0	25.7
	17.5	101.0
	21.4	37.8
	15.3	27.5
	15.7	24.5
	15.7	21.1
	15.7	23.1
	28.6	38.0
	18.7	21.5
	20.1	21.9
	19.1	28.2
	18.0	26.1
Avg	18.3 S.D. 3.4	31.1 S.D. 20.1

Depancreatized dogs without insulin treatment. Three to 5 days after withdrawal of insulin, with increasing glycosuria and ketosis, there developed a further steep rise of serum cholesterol and a moderate increase of lipid phosphorus, usually associated with visible lipemia. The degree of change seemed to depend upon degree of decompensation; with minor decompensation, *i.e.*, shortly after withdrawal of insulin in dogs with high insulin requirement or later in mildly diabetic dogs, the lipoprotein pattern maintained the same general features observed during the period of insulin control showing only a rather uniform rise of lipid content of all lipoprotein fractions. On the other hand, when decompensation was severe and ketosis marked, β-globulin fractions increased markedly, while albumin-α-globulin fractions either remained at the level reached at the beginning of decompensation or decreased. As severe decompensation set in, the average $\frac{\text{cholesterol}}{\text{lipid P}}$ ratio showed a further rise in all fractions, but more in β-globulin than in albumin-α-globulin fraction. In some decompensated dogs, an increase in the fraction faster than albumin was observed; in these cases, the fraction accounted for 5-20% of total serum cholesterol and lipid phosphorus.

Fig. 3 represents the correlation between the cholesterol content of the serum and cholesterol content of 2 main lipoprotein groups in normal and depancreatized dogs. Although the correlation between serum and albumin-α-globulin cholesterol is fairly good both in normal and controlled diabetic dogs, in decompensation, the rise in serum cholesterol is due mostly to a rise in the β-globulin fraction while the albumin-α-globulin fraction shows the irregularity of behavior noted above. When insulin treatment is restored, in a few days the picture returns to that of the control period. Typical course of events in a depancreatized animal with diabetes of moderate severity is represented in Fig. 4, in which the cholesterol content of the whole

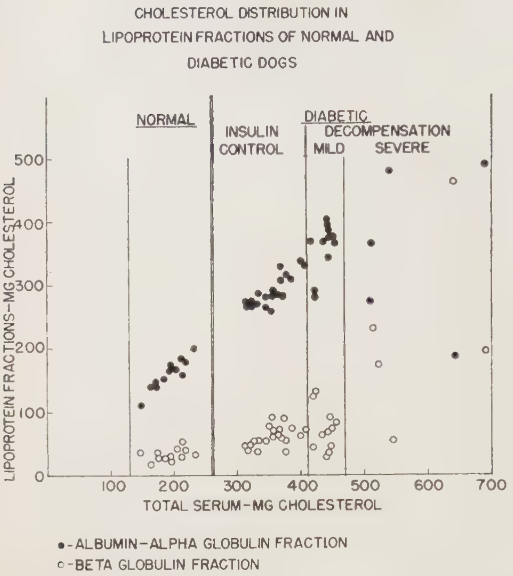


FIG. 3. Correlation between total serum cholesterol and lipoprotein cholesterol in normal and diabetic dogs.

serum and of the various fractions is being used to indicate the behavior of the lipoproteins.

Discussion. The results appear to indicate the following: 1. From the electrophoretic point of view, lipoprotein pattern of the normal dog consists of 3 major groups containing 5 fractions. The first group contains albumin and α-globulin and carries the largest proportion of serum lipids. The second group is that of β-globulins. This group normally has a low lipid content, but this increases after fat absorption. The third group is that of the β_2-γ-globulins which do not migrate at all. It is interesting to note that contrary to what happens in the normal dog, most lipids present in normal human serum are carried by the β-lipoprotein fraction and only a small amount by albumin-α-globulin fractions. 2. Moderate impairment of carbohydrate utilization, such as occurs in depancreatized dogs under insulin treatment, is associated with a rise of all lipoprotein fractions. This might indicate a "loading" of the fat transportation system, probably due to an increased fat mobilization. 3. When severe decompensation of diabetes sets in, the additional fat mobilized no longer can be handled normally and this is associated with a sharp rise of the β-lipoproteins. Perhaps this is due to an absolute or relative inadequacy of the factors involved in the conversion of the large molecule lipoproteins into smaller ones in the liver. 4. In the serum of diabetic dogs, there is a rise of the $\frac{\text{cholesterol}}{\text{lipid P}}$ ratio, the physiological implications of which are not clear at present.

The marked differences in the serum lipoprotein patterns of man and dog, demonstrated also by ultracentrifugal analysis(16), cautions against direct transfer of these results to human pathology. However, there are indications that an analogy of functions might exist between corresponding lipoprotein fractions in these two species: a) both fat absorption and heparin administration modify the same fractions in the serum of man and dog(15), and b) at high levels of serum lipids, under conditions suggesting an impairment of the normal conversion of lipoproteins, the β fractions become carriers of the bulk of serum lipids in both animals. The difference in relative proportions of various fractions, therefore, seems to be the result of

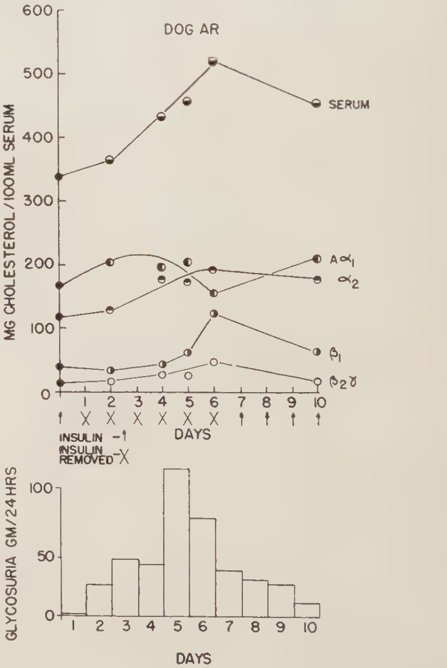


FIG. 4. Effect of insulin on serum lipoproteins in the depancreatized dog.

a different equilibrium and, probably, does not indicate a basic difference in the mechanism of lipid transport. For these reasons, it does not appear unreasonable to apply some of the findings obtained in the diabetic dog to diabetic man and suggest, tentatively, that a strict control of diabetes must be adopted to prevent those lipemic abnormalities suspected of having a pathogenetic relationship to atherosclerosis.

Summary. 1) Serum lipoproteins of normal and depancreatized dogs have been studied by means of zone electrophoresis on filter paper. 2) Dog serum contains 3 main groups of lipoprotein fractions: a fast-migrating albumin-α-globulin group, a slow-migrating β-globulin group and a β_2-γ-globulin group which does not migrate at all. 3) The bulk of serum lipids is carried by the albumin-α-globulin fractions and only a small amount by the β-globulins. This is the opposite of what happens in normal man. 4) Serum of depancreatized dogs under insulin control contains more total cholesterol and total lipid P than normal serum. The $\frac{\text{cholesterol}}{\text{lipid P}}$ ratio is increased. There is a tendency for an increase in amount of the slower migrating components of albumin-α-globulin fraction at the expense of the faster ones. 5) Withdrawal of insulin is followed by an immediate uniform increase of all lipoprotein fractions. As decompensation sets in, the β-globulin fraction increases out of proportion with other fractions. Thus, in decompensated diabetic dogs, as in man, the β-globulin fraction becomes the principal carrier of serum lipids. 6) The possible significance of these findings is discussed.

---

1. Fasoli, A., *Lancet*, 1952, v1, 106.
2. ———, *Acta Med. Scand.*, 1953, v145, 233.
3. Kunkel, H. G., and Slater, R. J., *J. Clin. Invest.*, 1952, v31, 677.
4. Fasoli, A., Ed. Premio Ganassini, Milano, 1953.
5. Fasoli, A., and Bonelli, M., *Minerva Med.*, 1951, v42 (2nd part), 402.
6. Snavely, J. R., Goldwater, W. H., Randolph, M. L., and Unglaub, W. G., *J. Clin. Invest.*, 1952, v31, 664.
7. Gofman, J. W., Jones, H. B., Lindgren, F. T., Lyon, T. P., Elliott, H. A., and Strisower, B., *Circul.*, 1950, v2, 161.
8. Keiding, N. R., Mann, G. V., Root, H. F., Lowry, E. Y., and Marble, A., *Diabetes*, 1952, v1, 434.
9. Hanig, M., and Lauffer, M. A., *ibid.*, 1952, v1, 447.
10. Foà, P. P., Weinstein, H. R., and Smith, J. A., *Am. J. Physiol.*, 1949, v157, 197.
11. Durrum, E. L., *J. Am. Chem. Soc.*, 1950, v72, 3043.
12. Flynn, F. V., and DeMayo, P., *Lancet*, 1951, v2, 235.
13. Bloor, W. R., Pelkan, K. F., and Allen, D. M., *J. Biol. Chem.*, 1922, v52, 191.
14. Horwitt, B. N., *ibid.*, 1952, v199, 537.
15. Fasoli, A., and Foà, P. P., unpublished data.
16. Page, J. H., Lewis, L. A., and Plahl, G., *Circul. Res.*, 1953, v1, 87.

Received January 20, 1954. P.S.E.B.M., 1954, v85.

Particle Size of Yellow Fever Virus. (20969)

ALFRED POLSON.

From the South African Council for Scientific and Industrial Research and the University of Cape Town Virus Research Unit, Department of Pathology, Medical School, Cape Town.

Since the original ultrafiltration experiments on yellow fever virus by Bauer and Hughes (1) and the ultracentrifugation studies on the virus by Pickles and Bauer(2) no work on the measurement of this virus has been reported.

Recently the technic of particle size determination using the Spinco preparative ultracentrifuge became available Polson and Linder(3). This method which allows sedimentation constants to be determined with fair accuracy and without laborious preliminary purification was used to determine the particle size of the yellow fever virus.

Material. The 17D strain of yellow fever virus was used after it had received 3 intracerebral passages in adult mice in this laboratory. The brains of 4 mice moribund after intracerebral inoculation of neurotropic yellow fever were emulsified in 40 ml 10% rabbit serum saline, the material was clarified by centrifugation at 2700 r.p.m. in a refrigerated centrifuge, and 6 ml of supernatant mixed with an equal volume of 2% Caminella sincta haemocyanine in phosphate buffer at pH 7.0. The haemocyanine tends to reduce sedimentation irregularities caused by convection (Polson and Linder l.c.). It was experimentally confirmed that haemocyanine does not interfere with sedimentation nor does it affect the viability of the virus.

Methods. Samples of the mixture of yellow fever virus and haemocyanine were ultracentrifuged in the model L Spinco centrifuge for 100 minutes at rotor velocities ranging from 11000 to 25000 r.p.m., and the virus contents at different levels in the tubes determined by titration in mice. Prior to sampling in each case the position of the haemocyanine boundary in the centrifuge tube was carefully noted. The samples were titrated by intracerebral inoculation of 10-fold dilutions into groups of 6 3-4 weeks old mice, each mouse receiving 0.03 ml of fluid. The mice were kept under observation for 2 weeks. Titres are expressed in LD_{50}. The titres of the different samples were plotted against their mean relative position in the centrifuge tube, and the position of the sedimenting boundary determined from the graph (Polson and Linder l.c.). The level of the sedimenting boundary was taken to be that at which the virus concentration was 50% of that of the original emulsion. The sedimentation constant of the virus was calculated from an equation deducted from that of Svedberg and Pedersen (4):

$$S_2 = S_1 \cdot \frac{H_2}{H_1} \cdot \left(\frac{2X_1 + H_1 \sin \alpha}{2X_1 + H_2 \sin \alpha} \right)$$

where S_2 and S_1 are the sedimentation constants of the virus and the haemocyanine and H_2 and H_1 the respective distances which their boundaries migrated down in the tube during centrifugation. X_1 is the distance of the fluid meniscus from the centre of rotation of the rotor, and α is the angle of inclination of the centrifuge tube with the vertical. The value of S_1 is 100 Svedberg units.

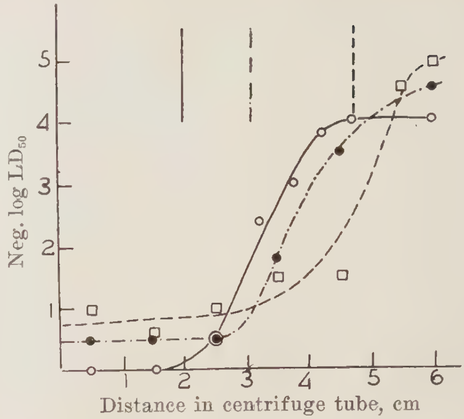


FIG. 1. Sedimentation diagrams of yellow fever virus, strain 17D, in 1% haemocyanine at pH 7.0. Vertical lines indicate position of haemocyanine boundary in different centrifugation runs. Values at 6 cm are titers before centrifugation. ——— = 18000 r.p.m. for 100 min. -•-•- = 19800 r.p.m. for 100 min. ---- = 24400 r.p.m. for 100 min.

Results. It was found that after centrifugation at 11000 r.p.m. for 100 minutes very little sedimentation of the virus had occurred and at rotor velocities of 25000 r.p.m. for the same period the virus was centrifuged down completely. At 18000 r.p.m. to 20000 r.p.m. the virus boundary was in suitable positions in the tube to allow calculation of the sedimentation constant. In Fig. 1 are given the sedimentation diagrams obtained at different rotor velocities together with the positions occupied by the haemocyanine in the different runs. It will be noticed that the virus boundaries were well ahead of the haemocyanine boundaries and that the upper layers of fluid in the centrifuge tube were almost completely freed of virus by the centrifugation. Table I records the results of the 3 experiments. The sedimentation constant calculated from equation (1) is 184 S for the run at 18000 r.p.m., and 157 S for that at 19800 r.p.m. From these values the particle size was calculated using the modified Stokes equation:

$$r^2 = \frac{9}{2} \frac{S\eta}{(d-\rho)}$$

TABLE I. Experimental Data of 3 Centrifugation Runs of Mixtures of Haemocyanine and Yellow Fever Virus Strain 17D. $a = 26°$. $X_1 = 5.32$ cm. $S_1 = 100$ Svedberg units.

r.p.m. (×100)	H_1, cm	H_2, cm	LD_{50} of samples taken at — cm										Original, 6 cm
			0.5	1.5	2.5	3.25	3.5	3.75	4.25	4.5	4.75	BL	
180	2.0	4.0	0	0	0–1	2.4	—	3.0	3.8	—	4.0	—	4.0
198	3.1	5.25	0–1	0–1	0–1	—	1.8	—	—	3.5	—	—	4.5
244	4.75	B	1	.6	1.0	—	1.5	—	—	1.5	—	4.5	4.9

B = bottom; BL = bottom layer.

where r is the radius of the particle, S the sedimentation constant, η the viscosity of water at 20°C, d the density of the virus particle assumed to be 1.33 g/cc and ρ the density of water at 20°C. The diameter of the particle determined in this way ranges from 29.2 to 31.4 mμ.

Discussion. The size of yellow fever virus particle as determined in this work is nearer that found by Bauer and Hughes (l.c.) in ultrafiltration experiments than that recorded by Pickles and Bauer (l.c.).

Summary. The particle size of the 17 D strain of yellow fever virus has been determined as ranging from 29.2 to 31.4 mμ by the method of ultracentrifugation coupled with biological assay.

The author wishes to express his gratitude to Professor M. van den Ende for his interest in this work, and to Miss T. Madsen for technical assistance. The virus used in this work was very generously supplied by Dr. J. H. S. Gear of the Poliomyelitis Research Foundation, Johannesburg.

1. Bauer, J. H., and Hughes, T. P., *Am. J. Hyg.*, 1935, v21, 101.

2. Pickels, E. G., and Bauer, J. H., *J. Exp. Med.*, 1940, v71, 703.

3. Polson, A., and Linder, A. M., *Bioch. et Bioph. Acta*, 1953, v11, 199.

4. Svedberg, T., and Pedersen, K. O., *The Ultracentrifuge*, Oxford, Clarendon Press, 1940.

Received March 4, 1954. P.S.E.B.M., 1954, v85.

Influence of Alpha-Tocopherol upon Development of Cardiovascular Necrosis and Hypertension in the Rat.* (20970)

David Lehr, Jacob Churg, and Robert Milora.

From the Department of Physiology and Pharmacology, New York Medical College, Flower and Fifth Avenue Hospitals, New York City.

Medical literature is replete with contradictory reports on therapeutic efficacy of vit. E. Thus large dosages of alpha-tocopherol or mixed tocopherols have been reported to exert therapeutic effects in muscular dystrophies and rheumatic diseases of men(1-3). Also that prolonged administration of this vitamin may bring about dramatic amelioration in hypertensive cardiovascular disease, as well as many peripheral vascular conditions(4-6). However, well controlled clinical studies by others failed to confirm the reported benefits(7-10). The vast animal experimental literature dealing with vit. E contains surprisingly few references to the beneficial action of this vitamin, if one takes exception to specific effects in induced deficiency. With regard to the cardiovascular system, only 2 publications described therapeutically desirable effects. Allardyce and associates(11) reported a hypotensive effect of vit. E in desoxycorticosterone induced hypertension of albino rats, and Holman(12) was able with the help of vit. E to inhibit emergence of experimental necrotizing arteritis induced in dogs on a "standard fat" diet by way of renal injury.

---

* This investigation aided by a grant from the National Heart Institute of the National Institutes of Health, U. S. Public Health Service.

In view of these latter reports and the controversial nature of clinical observations, it seemed of interest to evaluate the influence of vit. E upon development of hypertension and cardiovascular necrosis induced in the albino rat with the help of renal injury, using a short-term method developed in our laboratory(13).

Materials and methods. Closely bred 10-months-old albino rats of both sexes from our own colony were employed. Animals were maintained on Purina Chow. For the actual tests, subgroups of at least 5 rats each were placed on a special complete synthetic diet (14). Since cardiovascular necrosis and hypertension were produced by procedure previously described(13), only the main features of this method will be briefly restated. A single excessive dose of sodium acetyl sulfathiazole is injected intraperitoneally into adult albino rats. This induces severe "obstructive nephropathy" due to massive deposition of poorly soluble sulfonamide crystals in the renal tubules. Impairment of the kidney in turn results almost invariably in the development of extensive disseminated necrosis in the arterial tree as well as the myocardium, and frequently also, in the muscularis of the stomach within a period of 5 to 7 days. Necrosis is followed by a stage of reparative changes with scar formation and intense calcification. Marked hypertension developing about 5 days after renal block, lasting for 2 to 5 weeks, is a usual feature of the intoxication. It was calculated that control animals eating standard synthetic diet received about 0.8 mg of alpha-tocopherol daily. Experimental diet was enriched to yield a daily intake of 10 mg alpha-tocopherol[†] per rat. After 2 weeks, rats eating the enriched food received supplementary stomach tube feedings of alpha-tocopherol in olive oil (10 mg/100 g body weight), 3 times weekly for 4 weeks. This was to ensure an uninterrupted intake of alpha-tocopherol in excess, since spontaneous food consumption drops sharply after production of renal injury. Rats eating the normal synthetic diet received intubations of equal volumes of olive oil. Kidney damage was induced in both groups after third intubation. A total of 33 rats were employed. Of these, 2 groups of 5 animals each, served as normal diet and vit. E diet controls, respectively. They were not exposed to renal injury and were killed after completing the full course of alpha-tocopherol or olive oil intubation on the 29th day. In the experimental group exposed to renal injury and subsequent cardiovascular damage, 13 rats (8 ♂ and 5 ♀) were kept on alpha-tocopherol enriched regimen, whereas 10 rats (5 of each sex) were fed the control synthetic diet.

Food intake was determined daily and the body weight checked twice weekly. Systolic blood pressure was recorded at predetermined intervals with the help of a photoelectric tensometer which measures volume changes in the hind leg of unanesthetized rat(15). All measurements were made by one experienced experimenter. Except for an occasional moribund animal which was not subjected to the recording procedure, all control and experimental rats were measured at the same session. The mean of at least 3 to 5 consecutive satisfactory readings at intervals of several minutes was taken to represent the systolic blood pressure. The animals were killed at predetermined intervals by exsanguination in ether anesthesia. Blood was used for estimation of N.P.N., calcium, and inorganic phosphates. All rats, including those dying spontaneously as a consequence of renal injury, were subjected to complete postmortem examination. The heart, thymus gland, adrenals, and kidneys were weighed immediately after removal on a torsion balance to the nearest mg. These organs as well as the aorta and gastrointestinal tract, were examined under the microscope using routine and special stains.

Results. Data on the incidence and severity of the most important organic lesions found in experimental animals, after exposure to renal injury, are depicted in Table I and Fig. 1.

A glance at the life span of individual ani-

[†] Liberal supplies of alpha-tocopherol acetate were obtained through the courtesy of Dr. A. Pirk of Hoffmann-La Roche, Inc., Nutley, N. J.

TABLE I. Production of Disseminated Muscular Necrosis in the Albino Rat. Influence of excessive amounts of alpha-tocopherol.

	Heart, necrosis, calcification, cell infiltr.		Aorta,	Stomach,	Kidney,
Days killed or died* after renal block	Myocard.	Coron. vessels	medio-necrosis, perivasc. infiltration	necrosis and calcification, muscularis	pyeloneph. calcification, nephrosis
Alpha-tocopherol enriched synthetic diet					
Males					
6*	3+	3+	3+	+	4+
8*	2+	+	4+	3+	4+
9*	3+	3+	4+	—	3+
13*	3+	2+	4+	0	4+
16*	2+	3+	4+	0	2+
29	—	3+	2+	±	3+
29	—	2+	4+	2+	3+
29	—	2+	4+	3+	3+
Females					
7*	2+	3+	4+	0	3+
13*	2+		4+	0	3+
23	+	2+	4+	+	3+
23*	3+	3+	4+	—	4+
50	±	±	2+	—	3+
Normal synthetic diet					
Males					
7*	3+	+	±	—	2+
7*	±	—	—	—	2+
8*	2+	+	±	—	2+
62	—		3+	—	3+
62	—	+	—	—	3+
Females					
7*	3+	2+	3+	0	4+
13*	4+	2+	3+	0	4+
62	—	+	+	—	+
63	—	2+	4+	+	3+
50	+	2+	3+	—	4+

— = Normal organ (macroscopically and microscopically).
± = Definite but minor damage, recognizable under microscope.
+ = Considerable damage, recognizable under microscope.
2+ = Marked change, often visible to naked eye.
3+ = Advanced change, easily recognized upon gross inspection (except coronary vessels).
4+ = Severest degree of abnormality.
0 = Organ not examined.

mals (Table I) demonstrates that the extent of vascular, gastric or renal changes is not noticeably altered by variations in time of survival from 6 to >60 days following renal block. Myocardial damage, on the other hand, is most conspicuous within the first 2 weeks and shows a tendency towards complete healing in animals surviving 4 weeks or longer. Accordingly all 14 rats that died within the first 23 days, showed myocardial damage, usually of marked degree, whereas of the remaining 9 animals surviving more than 4 weeks only 2 manifested minor changes in the heart muscle. In view of the fact that most of these latter animals still had severe renal and vascular damage, it was assumed that they probably also had myocardial changes at an earlier date. This is supported by our findings in other recent studies with groups of rats killed within 14 days after renal block. As in the corresponding group of the present study, the incidence of typical myocardial alterations was usually close to 100%. It is obvious, therefore, that in comparative evaluation of changes in the heart, time elapsing between renal injury and death is of major significance.

With regard to changes in the muscularis

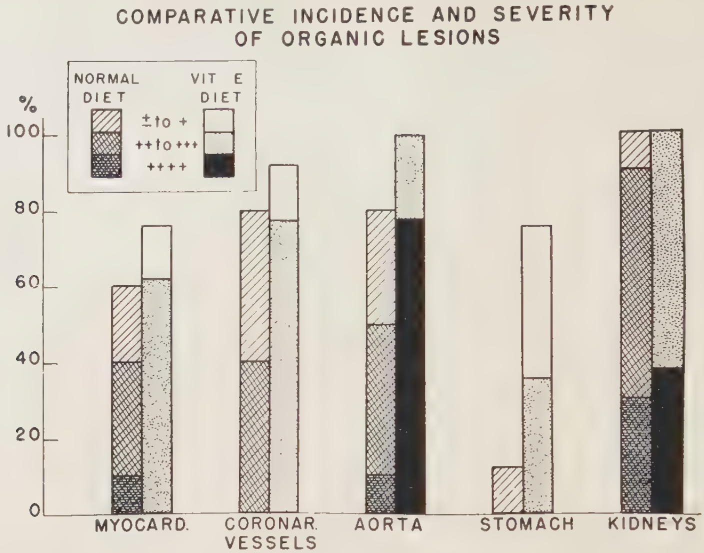


FIG. 1. Note substantial difference in *severity* of organic changes in coronary vessels, aorta and stomach of albino rats. (For explanation of + and — symbols see Table I.)

of the stomach it should be pointed out that advanced autolysis made it impossible to examine the stomachs of 6 rats. Of these, 2 were on control diet and 4 on vit. E enriched regimen. Nevertheless, the absence of any damage in the stomach wall of 7 out of 8 rats eating the normal diet, and the presence of necrosis in the muscularis of 6 out of 9 stomachs examined, from the vit. E group including 3 with advanced necrosis and calcification, adds considerable weight to the impression that damage of smooth muscles is enhanced in the presence of alpha-tocopherol in large amounts. This becomes particularly noticeable when the pathologic-anatomical alterations are grouped in accordance with the degree of damage (Fig. 1). Although, except for the stomach, total incidence of organic lesions is apparently not materially greater in rats receiving large dosages of alpha-tocopherol than in animals maintained on a normal diet, the distribution of coronary and especially aortic lesions according to extent of damage is highly suggestive of an aggravating effect of alpha-tocopherol.

Of the 5 control rats maintained on the alpha-tocopherol enriched regimen without being exposed to renal injury, 2 animals showed small foci of nephritis as the sole organic change. This insignificant lesion is not frequently observed as a spontaneous change in normal rats.

No substantial deviations from the norm were found in the blood calcium and inorganic phosphate levels of experimental animals, as determined in samples obtained at sacrifice, which meant at least 3 to 4 weeks after date of renal injury. The N.P.N. levels, on the other hand, were still elevated in many instances and remained above the normal range even after 50 and 60 days.

Following production of renal block, the rats of both diet groups suffered usual loss of body weight, about 20% after one week and more than 40% in 3 weeks. Thereafter, with increasing appetite, body weight usually returned to its initial value at about the fifth week. Control animals on both diets showed expected maintenance or slight gain in body weight.

Finally, the effect of alpha-tocopherol upon the systolic blood pressure of normotensive and hypertensive albino rats is depicted in form of a scatter-gram (Fig. 2). The figure contains individual blood pressure records of all 23 experimental rats on 5th and 3rd day before production of renal block and of survivors of this group on the 8th, 16th, and 22nd day after renal injury. In addition, this graph also contains blood pressure read-

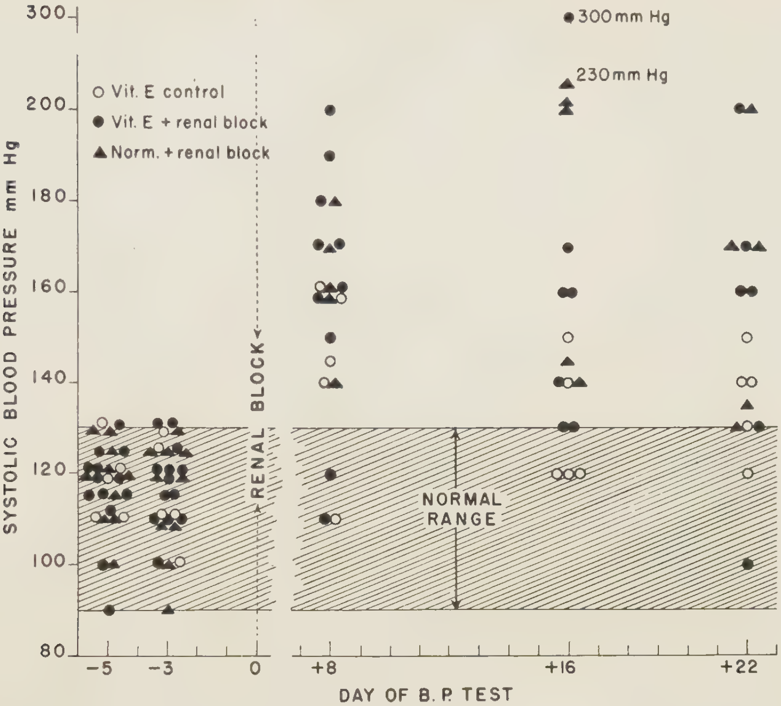


FIG. 2. Individual systolic blood pressures of 28 albino rats. In 23 of these rats a renal block was induced at zero day. The other 5 animals served as vit. E diet controls. Note marked hypertension resulting from renal injury and moderate elevation of systolic blood pressure caused by alpha-tocopherol administration.

ings of 5 control animals exposed to vit. E diet regimen. It is apparent that definite though moderately hypertensive levels were registered in some of the control rats on several occasions under the influence of alpha-tocopherol intubation (white circles). In contrast the blood pressure of 5 controls intubated with olive oil only, remained within normal range. (For the sake of greater clarity the blood pressure values of these latter controls were not included in the graph.)

Renal block, on the other hand, induced expected pronounced elevation of systolic blood pressure in the majority of experimental animals. No substantial difference in incidence or degree of hypertension could be derived from this small group of survivors feeding on either of the 2 diets. It is quite clear, therefore, that presence of abundant amounts of alpha-tocopherol in the body of the albino rat, does not noticeably interfere with the development of renal hypertension (black circles).

During the 14-day period of control observations before production of renal damage, systolic blood pressures of all experimental and control rats had kept well within normal range of 90 to 130 mm Hg.

Characteristic pathological anatomical changes in the cardiovascular system, gastrointestinal tract, adrenals, thymus gland, and kidneys were closely similar upon gross inspection and under the microscope to those observed in previous studies(14); also the change in moist weight of the 3 last named organs. The macroscopic and microscopic appearance of vascular lesions is illustrated in Fig. 3. Typical histological changes in the kidneys, the myocardium and the muscularis of the stomach are shown in Fig. 4. Lesions produced in presence of alpha-tocopherol in excess appeared to differ in degree of intensity rather than in quality from the organic damage induced in rats on a normal diet.

Comments. This study demonstrated that under the conditions of our experiments,

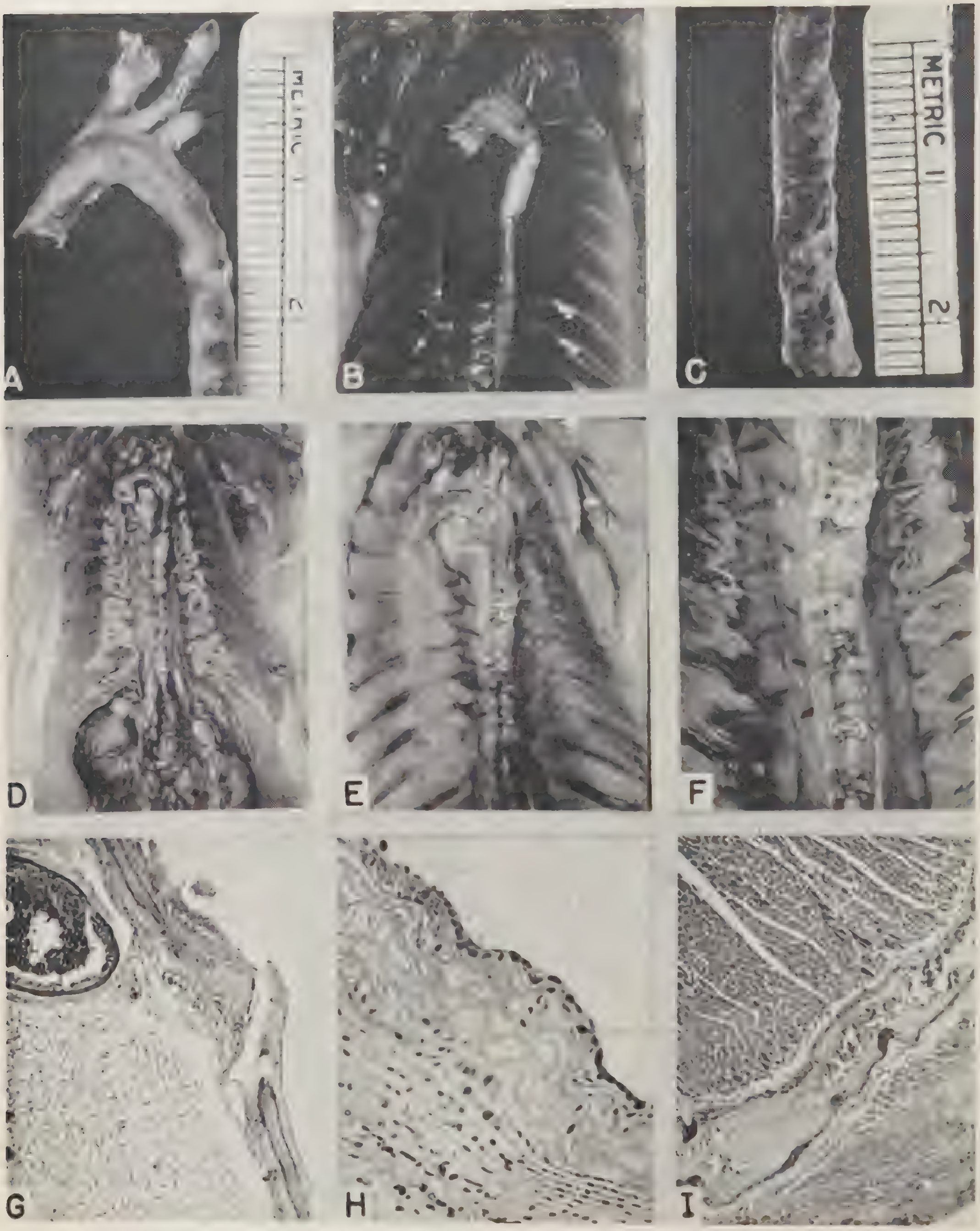


FIG. 3. Typical macroscopic and microscopic appearance of arterial lesions in alpha-tocopherol fed albino rats following renal injury. (Animals 1-13, Table I.)

A. Aortic arch and large branches. Bamboo-stick lesion of aorta extending into innominate, carotid and subclavian arteries.

B. Calcified aneurysm of aortic arch and upper part of descending aorta. Note normal thin-walled aorta below crossing of V. azygos. (Protection apparently due to mechanical pressure of vein.)

C. Abdominal aorta. Multiple aneurysmic dilatations of thinned out wall.

D. Dilation and bamboo-stick calcification of arch and descending aorta. Also visible are enlarged adrenal glands and contracted kidneys.

E. Open view of the aorta of Fig. D.

F. Same as Fig. E, close-up. Note irregular, bulging sub-intimal nodules. There is superficial resemblance to human atheromathosis.

G. Aorta and a large branch. (Left upper corner of photograph) showing necrosis and calcification of media.

H. Wall of aorta. Subintimal necrosis of media with swelling and breakdown of elastic membranes, and proliferation of endothelial cells over the lesion.

I. Longitudinal section of coronary artery. Wall is markedly thinned out, necrotic and irregularly calcified; lumen is filled with coagulated plasma and red cells.

alpha-tocopherol had no ameliorating influence upon the development of hypertension nor upon cardiovascular necrosis induced in the albino rat by way of renal injury. On the contrary, the presence of abundant supplies of this vitamin appeared to enhance muscular necrosis in the wall of blood vessels and stomach of these animals. In addition, alpha-tocopherol was found to be capable of producing moderate elevation of systolic blood pressure of normotensive control rats. These observations are at variance with the results of 2 experimental studies previously mentioned. Thus Allardyce and associates(11) reported that during daily administration of vit. E in 10 mg dosages (route of administration not stated), systolic blood pressure of rats, apparently stabilized at 300 mm Hg due to administration of desoxycorticosterone, showed a gradual decline to 250 mm Hg. However, only 6 rats were employed. Since only 3 of these animals received vitamin alone, whereas the remaining 3 were given histidine concomitantly, the significance of the observation is open to doubt. Our contrary finding of a blood pressure raising effect of vit. E is in line with the warning of Shute(16) that large dosages of alpha-tocopherol (300 mg or more daily) may cause a very considerable rise in both the systolic and the diastolic blood pressure of patients with hypertensive heart disease. It is also in conformity with the experimental data of Telford and associates (17) who reported about 30% reduction of systolic blood pressure in vit. E deficient rats as compared to normal control animals. Holman(12) who saw a preventive effect of vit. E in experimental arteritis induced in 8 dogs by "standard fat diet" and "standard renal damage," suggested that arterial lesions are induced by piling up of "toxic" substances (fatty acids) which are prevented from spilling over into urine when the safety valve of the kidney is destroyed.

The vascular lesions which we observed in albino rats were similar though far more severe than those seen by Holman. And yet, they developed in the absence of cholesterol and all animal fats from the diet of this species. Moreover, in experimental atherosclerosis caused by cholesterol feeding, vit. E was reported to have either no beneficial action(18) or to be responsible for accentuation of the characteristic arterial changes(19-21).

In trying to explain beneficial or detrimental effects of tocopherols, one becomes keenly aware that, among vitamins, vit. E in the physiology of the body is least well understood. It has been suggested, on the basis of knowledge of the effects of vit. E deficiency in the rat, that a major role of the vitamin *in vivo* is the control or restraint of one or more oxidative reactions, either by limiting the rate of some normally occurring reactions or by preventing the occurrence of detrimental reactions that take place only in its absence (22). It is well known that deprivation of this vitamin is followed by a bewildering array of functional and organic disturbances in different species of animals and that many of these disturbances can be cured in a striking manner by alpha-tocopherol administration (23). There should, of course, be no reason to assume that presence of large amounts of a substance must necessarily have a beneficial action upon tissues which suffer damage due to its depletion, and yet, judging from clinical reports, much of the reasoning in therapeutic application of vit. E seems to have proceeded in this manner.

In view of the many contradictory claims regarding the therapeutic capabilities of vit. E at the bedside, it would seem desirable that a more determined effort be made to distin-

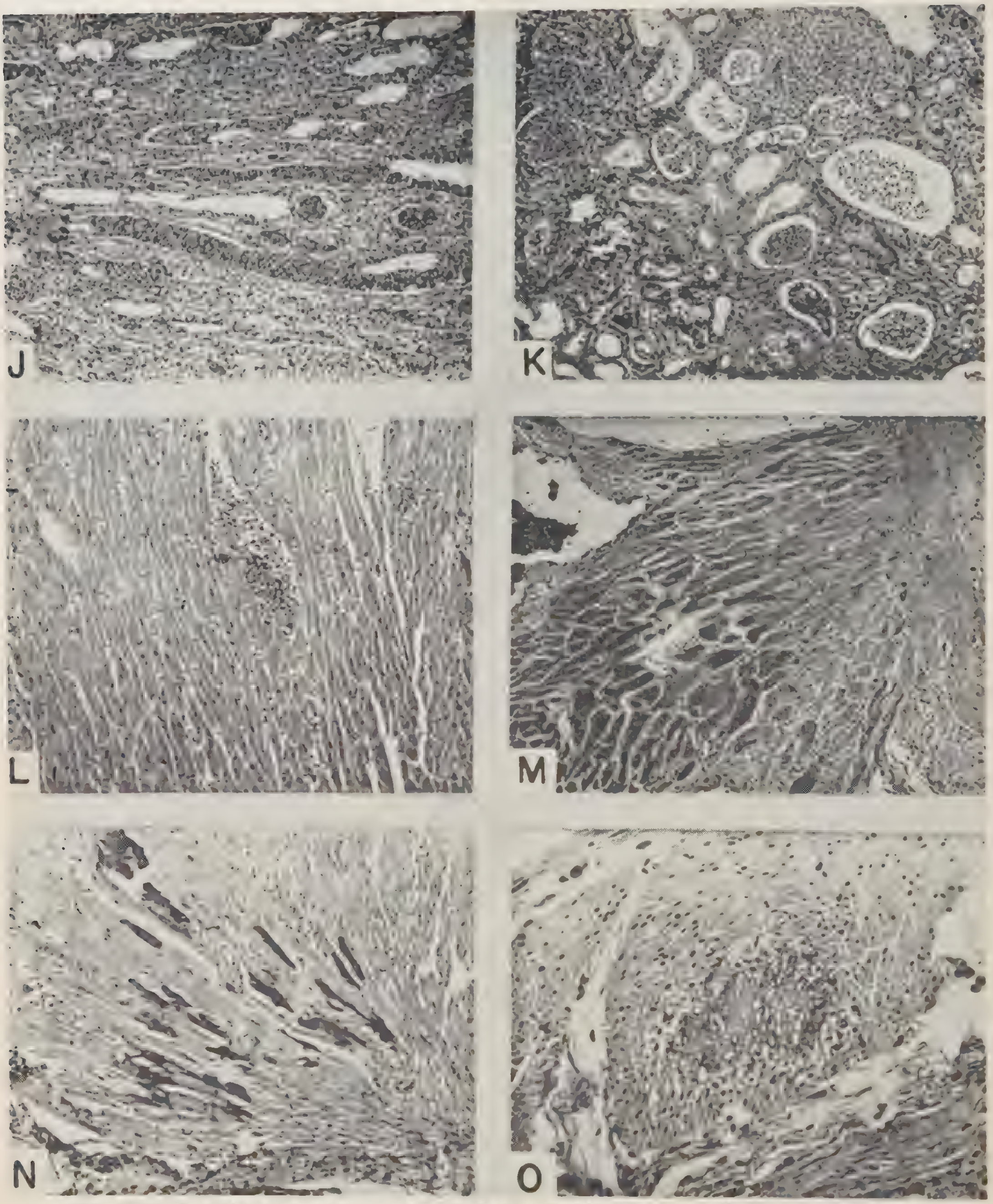


FIG. 4. Typical histological appearance of changes in kidney, myocardium and muscularis of stomach in alpha-tocopherol fed albino rats following renal injury. (Animals 1-13 of Table I.)

J. Ascending pyelonephritis in renal medulla. Note large tubular casts composed of polymorphonuclear leucocytes and interstitial infiltration.

K. Typical lesion of renal cortex. Showing tubular dilatation with casts of pus cells, interstitial inflammation, necrosis and calcification of tubular epithelium and foci of tubular regeneration.

L. Interstitial myocarditis. Diffuse infiltration and focal collection of predominantly mononuclear cells.

M. Focus of myocardial necrosis. Note darkly stained necrotic fibers in left lower corner of photograph.

N. Myocardium. Necrosis and calcification of individual muscle fibers.

O. Stomach wall. Showing necrosis and calcification in muscularis.

guish specific substitution effects in deficiency from pharmacodynamic effects obtained with relatively large dosages in the nondeficient organism. Most clinical applications of vit. E obviously belong to the latter group. In this connection it may be of interest to quote the chief proponents of vit. E therapy in cardiovascular disease(24): "Vit. E therapy in our hands is not substitution therapy but a form of chemotherapy. This is a point that deserves emphasis. The doses we use are larger than nutritional studies would demand, but usually smaller dosage is ineffectual. We think of alpha-tocopherol as a chemical compound which also happens to be a food constituent, but whose dosage level in established cardiac disease is not closely related to that coincidence. . . . A small dosage of E gives but little hint of what it can accomplish in massive dosage." We are in agreement and believe that our experimental studies have tested the drug effect of alpha-tocopherol in massive dosage, rather than a specific vitamin action.

In search for an explanation of the detrimental drug effect of alpha-tocopherol we were intrigued by the view of Lecoq *et al.*(25), who ascribe alkalizing properties to vit. E. They demonstrated that a permanent increase in nerve chronaxia due to hypervitaminosis E could be brought back to normal by acidification with the help of ammonium chloride. Since it was demonstrated by one of us(26) that ammonium chloride is capable of inhibiting and even preventing emergence of cardiovascular necrosis in the albino rat, we are now investigating the possibility that an alkalizing action of the vitamin might have been responsible for its deleterious influence in our experiments.

Summary. 1. Disseminated muscular necrosis following experimental nephropathy in albino rats maintained on complete rations, was not beneficially influenced by the presence of excessive amounts of alpha-tocopherol. On the contrary, this drug appeared responsible for a higher incidence of advanced lesions in the aorta, coronary arteries, and stomach. 2. Concomitant renal hypertension was not alleviated by the presence of alpha-tocopherol in excess. In normotensive rats large dosages of this compound may induce moderate hypertension.

---

1. Rabinovitch, R., Gibson, W. C., and McEachern, D., *J. Neurol. Neurosurg. and Psychiat.*, 1951, v14, 95.

2. Steinberg, C. L., *Ann. New York Acad. Sc.*, 1949, v52, 380.

3. Aut, M., *Rheumatism*, 1950, v6, 114.

4. Vogelsang, A., Shute, E., and Shute, W., *M. Rec.*, 1947, v160, 21, 91, 163, 230, 279.

5. Shute, E. V., Vogelsang, A. B., Skelton, F. R., and Shute, W. E., *Surg. Gynec. and Obst.*, 1948, v86, 1.

6. Shute, E., and Shute, W., *Am. J. Surg.*, 1952, v84, 187.

7. Baer, S., Heine, W. I., and Gelfond, D. B., *Am. J. M. Sc.*, 1948, v215, 542.

8. Levy, Hyman, and Boas, E. P., *Ann. Int. Med.*, 1948, v28, 1117.

9. Donegan, C. K., Messer, A. L., Orgain, E. S., and Ruffin, J. M., *Am. J. M. Sc.*, 1949, v217, 294.

10. A. M. A. Council on Pharmacy and Chemistry, *J.A.M.A.*, 1950, v142, 485.

11. Allardyce, J., Salter, J., and Rixon, R., *Am. J. Physiol.*, 1951, v164, 68.

12. Holman, R. L., PROC. SOC. EXP. BIOL. AND MED., 1947, v66, 307.

13. Lehr, D., and Churg, J., *J. Mt. Sinai Hosp.*, 1952, v19, 106.

14. Lehr, D., Churg, J., and Milora, R., *Bull. New York M. Coll.*, 1952, v15, 130.

15. Kersten, H., Brosene, W. G., Jr., Ablondi, F., and SubbaRow, Y., *J. Lab., and Clin. Med.*, 1947, v32, 1090.

16. Shute, W. E., *Ann. New York Acad. Sc.*, 1949, v52, 354.

17. Telford, I. R., Swegart, J. E., and Schoene, F. C., *Am. J. Physiol.*, 1945, v143, 214.

18. Dam, H., *J. Nutrition*, 1944, v28, 289.

19. Bruger, M., PROC. SOC. EXP. BIOL. AND MED., 1945, v59, 56.

20. Moses, C., Rhodes, G. L., and Levinson, J. P., *Angiology*, 1952, v3, 397.

21. Marx, W., Marx, L., Meserve, E. R., Shimoda, F., and Deuel, H. J., Jr., *Arch. Path.*, 1949, v47, 440.

22. Mackenzie, C. G., *Ann. New York Acad. Sc.*,

1949, v52, 202.

23. Miner, R. W., and Briggs, W., *ibid.*, 1949, v52, 63

24. Shute, W. E., Shute, E. V., and Vogelsang, A. B., *Ann. Int. Med.*, 1949, v30, 1004.

25. Lecoq, R., Chauchard, P., and Mazoné, H., *Ann. New York Acad. Sc.*, 1949, v52, 142.

26. Lehr, D., *Bull. New York M. Coll.*, 1943, v6, 113.

Received March 5, 1954. P.S.E.B.M., 1954, v85.

Inhibitory Effect of 5,6-Dimethylbenzimidazole and of 1,2-Dimethyl, 4,5-Diaminobenzene on Growth of *Mycobacterium tuberculosis*.* (20971)

LEONARD N. HALLINGER, ROBERT SILBER, AND GERTRUDE NEUMANN.
(Introduced by Robert G. Bloch.)

From the Pulmonary and Medical Divisions, Montefiore Hospital, New York City.

The incidence of pernicious anemia has been reported to be less in patients with active pulmonary tuberculosis than in the general population(1). It occurred to us that this observation might reflect vit. B_{12} synthesis by the infecting tubercle bacilli, with consequent masking of any clinical manifestations of pernicious anemia.† Advances in the application of biologic antagonism to anti-bacterial therapy led us to investigate the influence of vit. B_{12} fragments on the growth of *Mycobacterium tuberculosis*. The primary objective was to determine whether blockade of vit. B_{12} synthesis or utilization in the tubercle bacillus might deprive the organism of an essential metabolite and thus halt its growth.

Two compounds, 5,6-dimethylbenzimidazole and 1,2-dimethyl 4,5-diaminobenzene‡ have been described by some as precursors of vit. B_{12}(3), and by others as vit. B_{12} antagonists(4). The present report is concerned with preliminary observations of their effect on the growth of a virulent strain of *Mycobacterium tuberculosis* in Dubos Medium.

Method. Twenty screw-capped tubes, containing 6.1 ml of Dubos Medium and inoculated with roughly equal numbers of tubercle bacilli (0.1 ml of inoculum), served as controls. A similar quantity of such tubes, containing in addition, one of the various concentrations of 5, 6 D or of 1, 2 D, served as the experiment. All concentrations were simultaneously tested. In detail, actively growing, 6-day-old cultures of the H37Rv strain of *Mycobacterium tuberculosis*, which had been subcultured at least 3 times in Dubos' Tween-Albumin Medium(5), were employed as inoculum. Proportionate amounts of the basal culture medium and the inoculum, as well as the glucose and the bovine albumin, were pooled, to insure uniformity of concentration of the constituents in the many culture tubes. Subsequently, 5.1 ml of the suspension were added to each of the previously sterilized tubes, which contained either 1 ml of basal medium plus the compound tested or 1.1 ml of the basal medium for the control tubes. The final concentrations of 1, 2 D and of 5, 6 D were achieved by adding appropriate 0.1 ml aliquots of their aqueous solutions to lots of 20 tubes of inoculated medium. Because the 1, 2 D solution was found to be somewhat unstable, particularly when heated, it was sterilized by passage through a bacterial filter prior to the addition of the inoculum. The

---

* We express our gratitude to Dr. Robert G. Bloch, who gave generously of his advice and laboratory facilities, and to Dr. Raymond Weston and Dr. Louis Leiter for constructive criticism. This investigation was supported, in part, by a grant from the Minnie Wallach Memorial Fund. Dr. Karl Folkers, Merck & Co., Inc., Rahway, N. J., supplied 5, 6-dimethyl-benzimidazole and 1, 2-dimethyl 4, 5-diaminobenzene,—the culture of *Mycobacterium tuberculosis*, H37Rv, by Mr. William Steenken, Trudeau Laboratory, Trudeau, N. Y.

† More recent evidence has supported our hypothesis that vit. B_{12} may be synthesized by *Mycobacterium tuberculosis*(2).

‡ Hereafter referred to as 5, 6 D and 1, 2 D respectively.

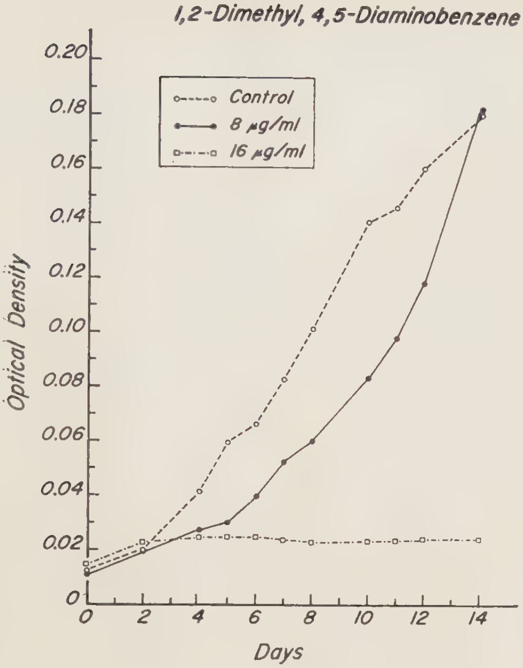


FIG. 1. Effect of 1, 2-dimethyl 4, 5-diaminobenzene, in various concentrations, on growth of *M. tuberculosis*, H37Rv, in Dubos Medium. Each plot on graph represents arithmetic average of 20 simultaneously growing cultures.

tubes were incubated at 37°C and shaken daily. Growth was determined turbidimetrically at 650 λ, with a Coleman Jr. Spectrophotometer, according to the method of Schaefer *et al.*(6). This was usually recorded daily. Each experiment was repeated at least 3 times.

Results. Fig. 1 illustrates a single typical graph of the effect of 1, 2 D on the growth of *Mycobacterium tuberculosis H37Rv*, expressed in units of optical density. It will be noted that with the lesser concentration, 8.0 μg/ml, there is partial inhibition of growth for the first 4 to 5 days. Doubling the quantity of 1, 2 D, 16.0 μg/ml, produces complete cessation of growth by the second to third day. The fluctuations thereafter are within the range of experimental error. Subcultures taken from these tubes on the twelfth or fourteenth days yielded viable organisms when cultured on Dubos Medium. Slightly higher concentrations were needed to inhibit growth when it was sterilized by Selas filtration rather than autoclaving.

A greater concentration of 5, 6 D was needed to achieve comparable inhibition (Fig. 2). With this compound viable organisms could not be subcultured at the end of the experiment.

Discussion. Antimetabolites are substances whose activity appears to be due to structural similarity between their molecules and those of biologically important substances such as vitamins, hormones, or amino acids. Because of this structural similarity, they may become incorporated into the metabolism of the organism at the reaction site(s) usually occupied by the metabolite they resemble. Their lack of biologic specificity prevents them from performing the exacting role usually required of the metabolite, and the reactions are blocked. These reactions are important in such functions as energy metabolism, growth, and reproduction, and the deficiency state thus produced may be manifested by cessation of growth or death of the organism.

The study of the metabolism of vit. B_{12} is comparatively recent. Degradation of this vitamin by acid hydrolysis has yielded 5, 6 D

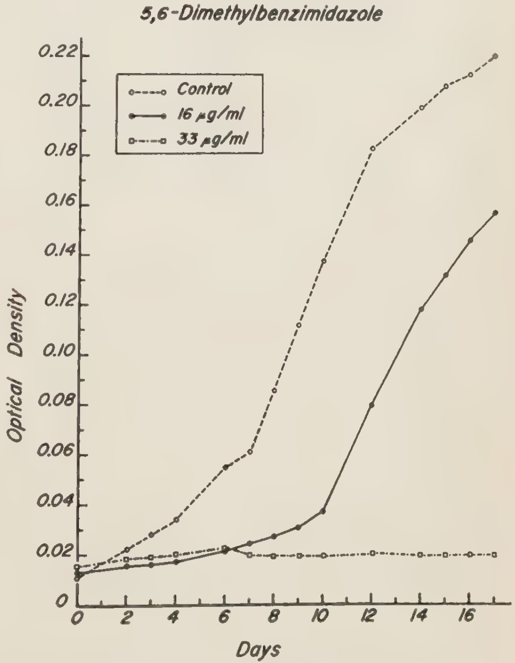


FIG. 2. Effect of 5, 6-dimethylbenzimidazole, in various concentrations, on growth of *M. tuberculosis*, H37Rv, in Dubos Medium. Each plot on graph represents arithmetic average of 20 simultaneously growing cultures.

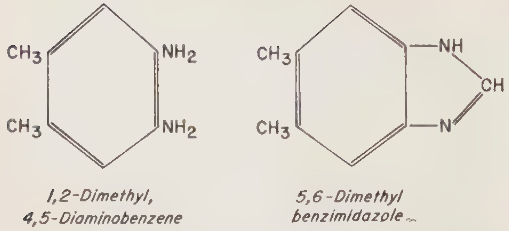


FIG. 3. An illustration of structural similarity between 1, 2-dimethyl 4, 5-diaminobenzene and 5, 6-dimethylbenzimidazole.

(7) and also a dimethylbenzimidazole riboside which on further breakdown was found to contain the 1, 2 D moiety(8) (Fig. 3). Certain observations with reference to these fragments have been made.

Woolley(3) believes that they are precursors of vit. B_{12}, and has demonstrated that 1, 2 D has the ability to counteract, in a competitive fashion, the toxicity of 1,2-dichloro 4,5-diaminobenzene which he had developed as an antimetabolite to both riboflavin and vit. B_{12}. Emerson *et al.*(9) have shown 1, 2 D and 5, 6 D to possess vit. B_{12} activity for the rat. Conversely, they have been thought to be vit. B_{12} antagonists by Hendlin and Soars who demonstrated growth inhibition of *Lactobacillus lactis Dorner* and other vit. B_{12} requiring organisms, with addition of 1, 2 D and 5, 6 D to the culture medium(4). The growth inhibitory effects of the benzimidazole nucleus (which is parent to both these compounds) has been adduced to operate in the area of vit. B_{12}-nucleic acid metabolism, since purines and vit. B_{12} can reverse this inhibition in some species of microorganisms(10).

While the evidence implicating these compounds in the metabolism of vit. B_{12} is indirect and circumstantial, the present report would seem to demonstrate yet another species in which vit. B_{12} is a metabolite, and in which these compounds produce a biologic effect. The results which we have obtained lead us to suspect that 1, 2 D and 5, 6 D may act as antimetabolites rather than precursors of vit. B_{12}, and either block its synthesis or compete for this vitamin's role(s) in the intermediary metabolism of the tubercle bacillus. The possibility also exists that these compounds are not active in the form wherein they were added to the culture medium, but rather underwent chemical change in the Dubos Medium to become a new substance, the active inhibitor.§ We recognize too, that another mechanism, yet unknown, may be the effective one. Further studies are planned in the hope of developing a new class of compounds of possible therapeutic value.

Conclusions. Two compounds, 5,6-dimethylbenzimidazole and 1,2-dimethyl 4,5-diaminobenzene, which have been previously implicated in vit. B_{12} metabolism, were found to inhibit the growth of *Mycobacterium tuberculosis, strain H37Rv,* in Dubos Medium.

---

§ 1, 2 D is known to be somewhat unstable in aqueous solution(11). The color change noted on autoclaving, and even at 37°C after 10 to 15 days in solution, perhaps results from a chemical reaction with one or more of the constituents of the culture medium. Some of the resultant products may possibly be similar to those synthesized by Woolley(12), with substituted or added alkyl groups on the parent nucleus. It may be that the superior inhibitory activity of 1, 2 D is due, in part, to one or more of these substituted compounds, rather than the parent compound. Since 1, 2 D and 5, 6 D are closely related (Fig. 3), it is probable that the mode of action of these and their questionably substituted forms are similar.

1. Baron, M., *J.A.M.A.*, 1933, v100, 1590.

2. Peterson, A., and Pope, H., *J. Bact.*, 1952, v64, 25.

3. Woolley, D. W., *J. Exp. Med.*, 1951, v93, 13.

4. Hendlin, D., and Soars, M., *J. Bact.*, 1951, v62, 633.

5. Dubos, R. J., and Middlebrook, G., *Am. Rev. Tuberc.*, 1947, v56, 334.

6. Schaefer, W. B., Marshak, A., and Burkhart, B., *J. Bact.*, 1949, v58, 549.

7. Brink, N. G., and Folkers, K., *J. Am. Chem. Soc.*, 1949, v71, 2951.

8. Brink, N. G., Holly, F. W., Shunk, C. H., Peel, E. W., Cahill, J. J., and Folkers, K., *ibid.*, 1950, v72, 1866.

9. Emerson, G., Brink, N. G., Holly, F. W., Koniuszy, F., Heyl, D., and Folkers, K., *ibid.*, 1950, v72, 3084.

10. Tamm, I., Folkers, K., Shunk, C. H., Heyl, D., and Horsfall, F. L., Jr., *J. Exp. Med.*, 1953, v98, 245.

11. Folkers, K., personal communication.

12. Woolley, D. W., and Pringle, A., *J. Biol. Chem.*, 1952, v194, 729.

Received March 11, 1954. P.S.E.B.M., 1954, v85.

Failure of Liver Feeding to Counteract Cortisone Effects Other than Growth Inhibition. (20972)

H. C. Stoerk, A. I. Knowlton, and E. N. Loeb.

From the Merck Institute for Therapeutic Research, Rahway, N. J., and the Department of Medicine, College of Physicians and Surgeons, Columbia University, N. Y. City.

Ershoff(1) reported that addition of 10% whole liver powder to an adequate synthetic diet minimized certain effects of oral cortisone acetate upon the immature rat. These effects included retardation of growth, alopecia and death. The present authors(2) reported an additional effect of cortisone acetate in rats, *viz.*, hypertension. The experiments herein described sought to determine whether addition of liver powder to the diet might also prevent development of hypertension as well as of other effects characteristic of prolonged cortisone administration.

Methods. Sixty-four male Sprague-Dawley rats were purchased from the Holtzman-Rolfsmeyer Company at 4 weeks of age. After a 2 week period of acclimatization the animals were divided into 8 groups (Table I). The animals in the first 4 groups were unoperated; bilateral adrenalectomies were performed at the outset of the experimental period upon rats in the other 4 groups. All animals were given a sodium-restricted synthetic diet of the following composition: Dextrose 60%, Labco casein 24%, Wesson oil 10%, cod liver oil 2%, sodium chloride-free USP salt mixture No. 2, 3.3%, and ammonium chloride 0.67%. This was supplemented with 10 g of the following vitamin mixture per kilo of diet: Vit. B_1 100 mg, Vit. B_2 200 mg, Vit. B_6 100 mg, pantothenic acid 1 g, nicotinic acid 1 g, inositol 500 mg, choline 10 g, PABA 3 g, biotin 5 mg, folic acid 20 mg, Vit. E 1420 mg, Vit. K 1420 mg, and Vit. B_{12} triturate 1 g. This basic diet was adjusted to contain 10% desiccated whole liver[*] for the rats in the 2nd, 4th, 6th and 8th groups. Cortisone acetate[†] was administered to all groups. In Groups III, IV, VII and VIII 3 mg of the steroid was injected subcutaneously daily in order to ensure both the control and liver supplemented groups receiving equal amounts despite possible variations in food intake. In Groups I, II, V, VI,

TABLE I. Effect of Dietary Liver Supplementation on Action of Cortisone Acetate upon Growth, Blood Pressure, Serum Sodium, Sugar and Liver Glycogen Levels.

Group:	I	II	III	IV	V	VI	VII	VIII
Adrenals:	intact				excised			
Cortisone acetate, mg/day	±6*	±6*	3	3	±6*	±6*	3	3
Route of steroid admin.	per os		subcut.		per os		subcut.	
Liver in diet, %†	0	10	0	10	0	10	0	10
Initial wt, g	118	118	118	118	118	118	118	118
Range	105–131	106–131	104–130	106–128	102–133	102–134	100–132	104–134
Final wt, g	123	155	118	112	103	109	106	104
Range	97–142	137–181	110–131	100–125	90–120	92–128	85–115	92–118
Change in 14 days	+5	+37	0	–6	–15	–9	–12	–14
Initial blood pressure, mm Hg	112	110	115	105	115	117	113	112
Final blood pressure, mm Hg	170	173	171	169	189	174	179	173
Terminal serum Na,‡ mEq/L	148.	143.8	140.5	141.5	130.0	132.0	142.0	141.5
Terminal serum sugar,‡ mg %	99	215	285	283	95	132	270	130
Liver glycogen,§ mg/g	7.6	10.7	46.0	48.0	7.9	8.5	49.5	52.0

* Steroid incorporated in food conc. of 6 mg/10 g of diet. † Armour's desiccated liver powder.
‡ Avg of 2 pools of 4 individual sera. § Avg of 2 determinations each based on pool of 4 livers.

---

[*] Armour and Co.

[†] Cortone Merck.

however, the steroid was incorporated into the food, as described by Ershoff(1), in a concentration to provide 6 mg/10 g of diet. Body weights were recorded daily. Blood pressure determinations(3) were made without anesthesia, at the outset and at the conclusion of the experimental period of 2 weeks. Prior to sacrifice food was withheld from all animals for 16 or 24 hours. Then the rats were anesthetized with nembutal and blood pipetted from the incised heart for determination of serum sugar and sodium concentration.‡ Sera were collected individually, and pooled from 4 animals, providing 2 separate pools in each group. At autopsy, body weight, and weights of heart, adrenals, thymus, right kidney, testicular fat body, and liver were recorded. The livers were then collected in dry ice in two pools from each group and total reducing substances (liver glycogen) determined. Chemical and bioassays were carried out on mixtures of liver powder with cortisone acetate.§

In addition to the above experiment, additional data were accumulated on body growth and adrenal weight of groups of 6 to 12 normal rats receiving a low sodium diet(2) with 10% liver supplementation and cortisone acetate orally in a concentration of 2 mg/100 g diet, or subcutaneously in a dosage of 2.0, 1.0 and 0.5 mg daily. The results, combined with data from Groups II and IV in Table I, are presented in graphic form in Fig. 1.

Results. The results are set forth in Tables I and II and in Fig. 1. In the first table, 4 observations relative to growth are notable: 1) Comparison of the weight gains of rats in Group II, given dietary supplementation with liver powder, with those of Group I, indicates that the growth-inhibiting effect of the steroid was prevented by the liver as reported by Ershoff; 2) when the steroid was injected subcutaneously, however, it was seen that the addition of liver to the diet failed to counteract growth retardation (*cf.* Groups III and IV); 3) the addition of liver to the diet was

TABLE II. Effect of Dietary Liver Supplementation on Action of Cortisone Acetate upon Body and Organ Weights.*

Group:	I	II	III	IV	V	VI	VII	VIII
No. in group	8	8	8	8	8	7	8	8
Adrenals	intact				excised			
Cortisone acetate, mg/day	±6	±6	3	3	±6	±6	3	3
Route of steroid admin.	per os		subcut.		per os		subcut.	
Liver in diet, %†	0	10	0	10	0	10	0	10
Body wt, g	123	155	118	112	103	109	106	104
Range	97–142	137–181	110–131	100–125	90–120	92–128	85–115	92–118
Adrenals, mg	22(18)	21(14.9)	18(15.3)	17(14.2)				
Range	16–37	13–26	16–21	16–20				
Heart, mg	635(516)‡	693(446)	667(565)	678(605)‡	603(585)‡	609(560)	627(590)	615(592)
Range	562–696	638–743	555–715	593–785	554–660	553–690	512–690	553–650
Right kidney, mg	638(518)	739(477)‡	665(563)	713(637)	592(574)	684(629)§	650(614)	644(619)
	545–784	616–820	534–731	603–811	522–650	610–753	513–795	538–731
Thymus, mg	52(42)‡	61(39)	21(18)	23(21)‡	32(31)	36(33)	17(16)	19(18)
	34–70	37–96	14–34	14–30	22–42	28–61	10–28	11–28
Testicular fat body, mg	615(500)	971(626)	895(759)	866(774)	514(499)	472(432)	723(681)	634(609)
	420–962	768–1204	521–1250	510–1156	379–676	397–620	462–856	522–699
Liver, g	4.43(361)	5.61(362)	5.46(464)	5.96(532)	3.91(380)	3.88(356)	5.49(517)	5.55(534)
	3.56–5.05	4.82–6.21	4.40–6.55	4.74–7.61	2.95–4.57	3.77–5.50	4.39–6.21	2.95–4.57

* Values in parentheses = wt/100 g body wt. † Armour's desiccated liver powder. ‡ 7 animals. § 6 animals.

---

‡ For these determinations we are indebted to Dr. D. M. Tennent.

§ For these assays and for the blood sugar and glycogen determinations we are indebted to Dr. C. C. Porter and Dr. R. H. Silber.

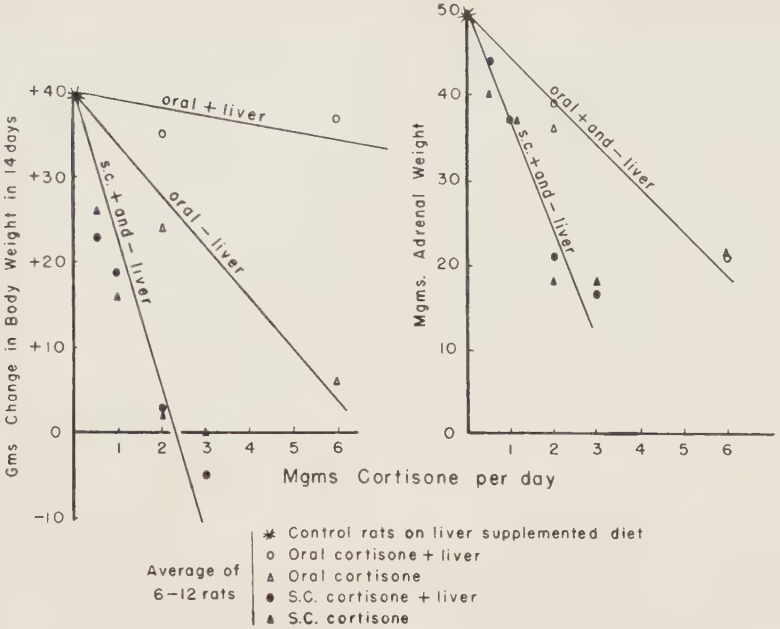


FIG. 1.

Left—Ability of liver to counteract growth retardation due to orally administered cortisone.
Right—Failure of liver to counteract adrenal atrophy due to orally administered cortisone.

without effect upon the growth of adrenalectomized rats given cortisone acetate regardless of the route of administration of hormone (compare Groups V through VIII). 4) Comparison of Groups I and II, neither receiving liver supplement, indicates that the steroid was much more effective in suppressing body growth when given parenterally than orally, since equivalent degrees of suppression were obtained with parenteral doses one-half as large as the oral doses.

Determinations of blood pressure reveal that a definite and comparable degree of hypertension developed in all animals by the end of the 2-week experimental period. It is noteworthy that the elevations in blood pressure in the Group II rats, in which the addition of liver prevented the suppressive effect of cortisone acetate upon growth, were quite as striking as in the other 7 groups.

The terminal serum sodium concentrations were essentially normal except in Groups I, V and VI. In the first group the concentration was abnormally elevated, while in the fifth and sixth groups the levels were significantly reduced. However, levels of serum sodium in adrenalectomized groups receiving liver were comparable to those of rats on the unsupplemented diet. Since the serum sodium level in adrenalectomized rats on low sodium diets bears a relation to the dosage of cortisone(4) the findings suggest the absorption of equivalent amounts of cortisone whether or not the diet was supplemented by liver.

Serum sugar values were definitely elevated in 3 of the 4 groups receiving parenteral cortisone acetate, while this obtained in only one of the 4 groups receiving the steroid orally. Similarly the glycogen content of the livers was markedly greater in the rats receiving parenteral hormone. Again liver failed to counteract the expected rise in blood sugar and liver glycogen due to cortisone.

Organ weights are presented in Table II. In the first 4 groups (animals with intact adrenals), marked adrenal atrophy occurred following administration of cortisone by either route irrespective of whether the diet was supplemented with liver. Other organ weights, especially when expressed in relation to body weight, give evidence of a slightly greater hormonal effect in groups receiving parenteral steroid even though the administered dose was approximately one-half that given the other animals, *i.e.,* the hearts and kidneys in these groups were heavier as were the testicular fat and liver weights, and the thymus was more strikingly atrophied. None of the anatomical changes typical of cortisone action were reversed by liver feeding.

Data presented in Fig. 1 illustrate that in animals receiving the liver supplement cortisone acetate had no effect upon body growth, whereas when the parenteral route was used, increasing doses of cortisone were accompanied by progressively more severe growth retardation even though the diet contained the same liver supplement. In contrast to the marked differences in the effect of liver supplementation upon growth response to orally and parenterally administered hormone, analogous and progressively more severe degrees of adrenal atrophy obtained in all animals as the dosage increased, regardless of the route of administration of the hormone.

Discussion. In view of the finding that liver feeding failed to alter any of the actions of injected cortisone but did abolish its growth depressing action when the steroid was given orally, it was possible that cortisone may be directly decomposed by some factor in liver. To test this, known quantities of cortisone were mixed with liver powder, re-extracted and assayed chemically (Porter, Silber reaction) and biologically (glycogen deposition in mice). By these methods, undiminished quantities of cortisone could be recovered from the mixtures. Hence a direct action of liver powder upon the steroid appears excluded. It remains possible that some factor is added by the rat's intestinal tract which mediates such an action of liver. This appears unlikely, however, since cortisone fed rats, in which the expected growth retardation was counteracted by addition of dried liver to the diet, exhibited other characteristic effects of chronic steroid administration. Equivalent degrees of hypertension, and adrenal and thymic atrophy occurred in these animals as in rats receiving no liver supplement. Hence the action of liver on body weight gain of cortisone-treated rats does not appear to be part of an over-all diminution in hormonal action.

Ershoff reported that in addition to counteracting growth suppression of cortisone acetate, the addition of liver powder to the diet also counteracts retardation of growth and decreases the mortality which accompanies a variety of unfavorable experimental conditions, *e.g.,* exposure to cold(5), sublethal doses of Roentgen rays(6), exhaustion induced by swimming in cold water(7), effect of drugs such as atabrine(8) or dinitrophenol (9), as well as of hormones such as thyroid (10) and cortisone.

Although there are multiple growth promoting factors in liver powder, among which may be nonspecific appetite stimulating substance, it is perhaps pertinent to contrast the "liver effect" in animals receiving cortisone with that of a single growth promoting substance, somatotropin. The antagonism of growth hormone is evident not only upon body growth but, with the exception of adrenal atrophy, also upon every one of the anatomical changes induced by parenteral administration of cortisone(11,12). This is in contrast to the action of desiccated liver which diminishes none of the cortisone effects except that on growth. Ershoff(13) reports an additional dissimilarity in that growth hormone, unlike liver powder, exerts no effect on growth in animals exposed to cold.

The reason for the absence of the "liver effect" in adrenalectomized rats remains unknown. Since the presence of cortisone did not permit this effect in adrenalectomized animals, it is possible that the activity of other steroids from the adrenals, presumably those of the sodium retaining group, is essential for the "liver effect."

Finally, these studies reveal a marked disparity in the effectiveness of oral and parenteral cortisone acetate in the rat. This has been previously observed by others(14). Even though the oral dose was approximately double that given parenterally, less marked changes occurred with oral administration.

Conclusion. 1. Desiccated liver powder as a dietary supplement to a low sodium diet is effective in counteracting the growth inhibition induced by cortisone acetate in rats only if the steroid is presented orally. It is without demonstrable effect in animals receiving the hormone by injection. It is also without effect in adrenalectomized animals, irrespective of the route of hormone administration. 2. The prevention of growth inhibition by liver powder is not accompanied by other evidences of diminished cortisone activity, *i.e.*, despite normal growth such animals exhibit physiological, anatomical and biochemical changes of the same degree as do cortisone treated rats without liver supplement.

The authors wish to express their appreciation to Tatiana Budzilovich, Theodore Bielinski and Dorothy Wendelle for technical assistance.

1. Ershoff, B. H., PROC. SOC. EXP. BIOL. AND MED., 1951, v78, 836.

2. Knowlton, A. I., Loeb, E. N., Stoerk, H. C., White, J. P., and Heffernan, J. F., *J. Exp. Med.*, 1952, v96, 187.

3. Friedman, M., and Freed, S., PROC. SOC. EXP. BIOL. AND MED., 1949, v70, 670.

4. Knowlton, A. I., Loeb, E. N., Stoerk, H. C., and Mohler, W. C., *J. Clin. Invest.*, 1953, v32, 1175.

5. Ershoff, B. H., and Williams, H. B., PROC. SOC. EXP. BIOL. AND MED., 1950, v75, 226.

6. Ershoff, B. H., *J. Nutrition*, 1952, v47, 289.

7. ———, PROC. SOC. EXP. BIOL. AND MED., 1951, v77, 488.

8. ———, *J. Nutrition*, 1948, v35, 269.

9. ———, *ibid.*, 1950, v42, 271.

10. ———, PROC. SOC. EXP. BIOL. AND MED., 1947, v64, 500.

11. Becks, H., Simpson, M. E., Marx, W., Li, C. H., and Evans, H. M., *Endocrinol.*, 1944, v34, 311.

12. Stoerk, H. C., unpublished observations.

13. Ershoff, B. H., *Endocrinol.*, 1951, v48, 111.

14. Silber, R. H., and Porter, C. C., unpublished data.

Received March 16, 1954. P.S.E.B.M., 1954, v85.

In vitro Effect of Autologous Lipemic Plasma on Platelet Suspensions.* (20973)

CHARLES W. LOUGHRY AND JACK W. COLE.

From Surgical Research Laboratories, Western Reserve University School of Medicine and Department of Surgery, University Hospitals of Cleveland.

It has recently been observed that hyperlipemia, produced by orally or intravenously administered fat emulsion, results in a marked decrease in the number of circulating blood platelets(1). As yet, there is no satisfactory explanation for this phenomenon. It has been postulated that this may be due to intravascular agglutination of platelets. This possibility suggested itself when it was noted that the intravenous injection of heparin during the hyperlipemia resulted in a prompt return of the platelet count toward normal values. This finding offers substantial evidence that platelets are not destroyed during intervals of hyperlipemia but rather supports the concept that they are "clumped" and that heparin promotes redispersion.

Swank's(2) observation that hyperlipemia produced agglutination of red blood cells led us to investigate the possibility that a similar process might prevail with platelets. The following experiments were carried out to determine whether or not platelet suspensions would demonstrate agglutination when incubated *in vitro* with autologous lipemic plasma.

Methods. All glassware used in these experiments was siliconated. A platelet suspension was prepared from 150 cc of whole blood obtained from normal healthy adult males according to the method of Stefanini(3) with minor modification. The donors were then given 250 cc of intravenous fat emulsion† over a 2-hour period. Platelet counts (Rees-Ecker) were obtained on the donor's blood prior to the administration of the Lipomul and at varying intervals during its administration. When a significant fall (40-50%) in the individual's platelet count was noted, a 10 cc blood sample was obtained. The blood was centrifuged and the supernatant plasma removed.

---

* Aided by a grant from the Cleveland Area Heart Society.

† Lipomul—supplied by Dr. Robert Heinle of the Upjohn Co.

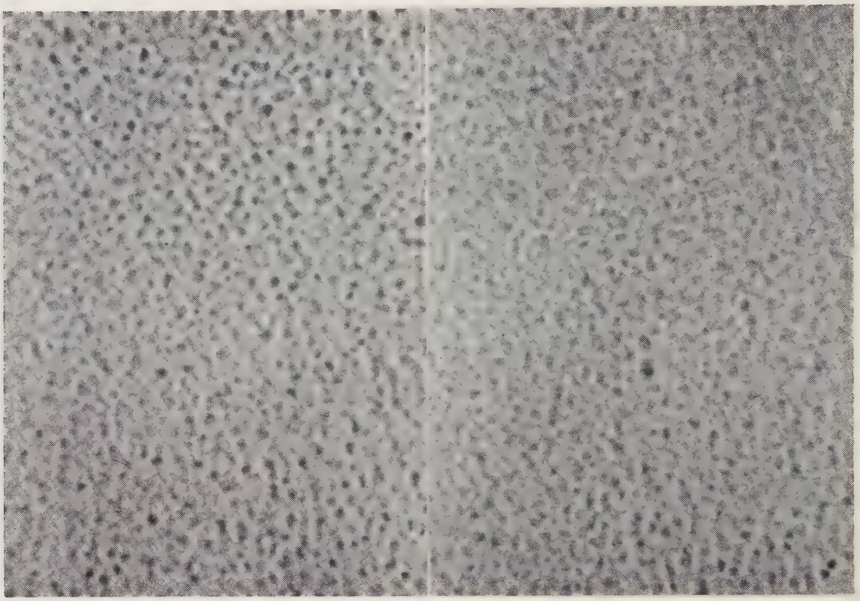


FIG. 1. Microscopic appearance, after 16 hr of incubation, of platelet suspensions mixed with autologous lipemic plasma (left) and physiological saline (right). (× 322)

One drop of the donor's previously prepared platelet suspension and one drop of the donor's hyperlipemic plasma were then mixed on a glass slide with a cover slip and sealed with paraffin. A similar preparation was made mixing saline and the platelet suspension for a control. Several such preparations were made for study. The slides were then incubated at 37°C for 16 hours and examined microscopically. In addition, siliconated stoppered test tubes containing 1 cc of the platelet suspensions and 1 cc hyperlipemic plasma and/or saline were incubated and observed microscopically after a 16-hour incubation period at 37°C.

Results. After the administration of 250 cc of intravenous Lipomul to one normal adult the platelet count fell from 460000/cu mm to 265000/cu mm, thus establishing that in this individual the hyperlipemia had produced a significant decrease in the circulating blood platelets. Similar results were noted in 4 additional subjects. However, when the plasma which was obtained at the time of the maximum platelet depression was incubated with a platelet suspension previously prepared from the same individual no agglutination was noted during a 16-hour period of observation, in either the slide or test tube preparations. The platelets were evenly dispersed and no significant morphological alterations were apparent in either the experimental or control mixtures. Representative fields are shown in Fig. 1. Identical results were obtained in 10 such experiments.

Summary. 1. *In vitro* mixing and incubation of blood platelets and autologous lipemic plasma does not result in apparent platelet agglutination.

The authors wish to express their appreciation for the technical assistance of Mrs. Eleanor Connor and Miss Alice Melcher.

---

1. Cole, J. W., Britt, A. G., Loughry, C. W., and Holden, W. D., in press.

2. Swank, R. L., *Am. J. Physiol.,* 1953, v164, 798.

3. Stefanini, M., Dameshek, W., Chatterjea, J. B., Adelson, E., and Mednicoff, I. B., *Blood,* 1953, v8, 26.

Received March 22, 1954. P.S.E.B.M., 1954, v85.

Relative Rates of Biological Decay of Thyroidal I^{131} in Rat and Guinea Pig.[*] (20974)

CATHERINE E. STEVENS AND S. A. D'ANGELO.

From the Daniel Baugh Institute of Anatomy, Jefferson Medical College, Philadelphia, Pa.

In the past a number of qualitative and quantitative estimates of thyroid gland activity has indicated that there is a considerable species difference between the rat and the guinea pig in the rate of production, secretion and utilization of thyroid hormone. Most parameters of thyroid function commonly measured (*e.g.* 24-hour I^{131} uptake, serum P.B.I., gland morphology) give a static picture of thyroidal activity rather than the dynamic one provided by measurement of rates of its biologic processes. In this work a modification of published technics(1-4) for measuring the I^{131} content of the thyroid *in vivo* over a period of weeks after injection has been applied to the normal rat and guinea pig. The results are used to estimate the rate of release of radioiodine from the thyroid which is considered to be a reflection of the tempo of hormone secretion(1-4).

Methods. The animals used were adult female guinea pigs (500-600 g body weight) fed *ad libitum* on a diet of Purina Omolene (Iodine; 1 μg/g)[†] and Rabbit Chow Checkers (Iodine; 5.2 μg/g),[†] tap water and fresh greens, and adult female albino rats (200-300 g body weight) of the Wistar strain, fed on Purina Dog Chow Checkers (Iodine; 1.5 μg/g)[†] and tap water. A single dose of carrier-free radioiodine (I^{131})[‡] was injected intraperitoneally into guinea pigs (25-40 μc) and rats (25 μc). The radiation-detection apparatus consisted of an El-Tronics scaler and Tracer Lab TCG-8 stainless steel gamma sensitive Geiger-Muller tube. The tube was placed vertically with end-window facing upwards, and housed in a cylindrical lead jacket with walls of 20 mm thickness. A lead shield 12 mm thick covered the end of the tube except for a central area 22 mm in diameter which was bridged with cellophane tape. The upper surface of the shield was flush with a board on which the animal (with shaved neck) was placed prone after ether anaesthetization. The radioactivity of the thyroid region was measured for at least 1000 counts at several positions over the Geiger tube. The highest count was used. Such counts were made on 16 rats at 4 hours after I^{131} injection and once daily for 8 days. For 16 guinea pigs, counts were made at 4, 24, 48 and 72 hours after injection and at 3-4-day intervals thereafter over a period of approximately 3 weeks. Extrathyroidal radioactivity was measured over the thyroid and epigastric regions of thyroidectomized guinea pigs. The ratio of neck to epigastric counts was found to be 0.37 at 4 hours, 1.0 at 24 hours, and 1.4 at 2-6 days at which time radioactivity became negligibly low. Counts over the epigastrium of normal guinea pigs multiplied by these ratios gave values for the extrathyroidal radioactivity in the neck region. For rats, one half the epigastric count

TABLE I.
Loss of Radioactivity from the Thyroid.

Days after zero time*	Guinea pig	Rat
1		(16)[†] 78.4 ± 2.7[‡]
2	(8)[†] 94.0 ± 3.6[‡]	(16) 60.6 ± 2.7
3		(16) 55.6 ± 3.3
4	(8) 85.0 ± 2.9	(16) 48.2 ± 3.2
6	(8) 83.9 ± 2.6	(15) 36.3 ± 2.5
7	(8) 80.4 ± 1.8	(14) 33.4 ± 3.0
8		(10) 31.5 ± 3.7
10	(12) 73.1 ± 1.9	
11	(7) 77.9 ± 1.7	
14	(7) 74.2 ± 4.7	
15	(12) 71.6 ± 2.8	
18	(7) 59.3 ± 3.1	
19	(12) 61.1 ± 4.3	

* Zero time = Time after injection at which maximal radioactivity (100%) was found in the thyroid.

† No. of determinations.

‡ Mean I^{131} content in thyroid as % of that at zero time ± stand. error of the mean.

---

[*] Supported, in part, by research grant, from the National Institute of Arthritis and Metabolic Diseases, Public Health Service and by funds under contract with the USAF School of Aviation Medicine, Randolph Field, Texas.

[†] Commercial estimate.

[‡] I^{131} was obtained from the Atomic Energy Commission, Oak Ridge, Tenn.

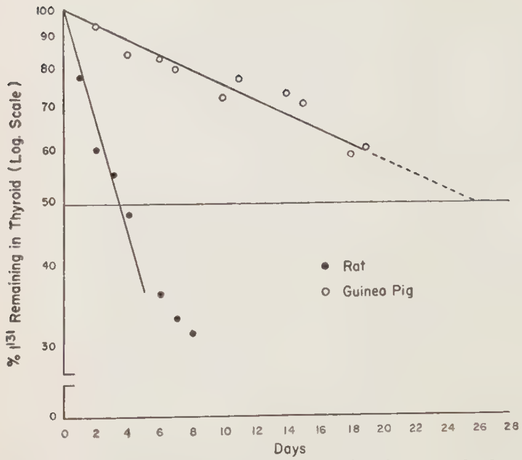


FIG. 1. Relative rates of biologic decay of thyroidal I^{131} in rat and guinea pig.

was used(1). The total radioactivity of the neck region was then corrected for these extrathyroidal values as well as for isotopic decay to give the theoretical thyroidal count.

Results. Maximal uptake of radioiodine by guinea pig thyroids occurred between 48 and 72 hours and was followed by a slow progressive decline. In rats maximal uptake was usually attained by 24 hours. Inasmuch as there was considerable variation from animal to animal in degree of uptake, counts at 24 and 48-72 hours after injection for rats and guinea pigs respectively were expressed as 100% for each animal with subsequent values as a percentage of this maximum (Table I). The mean percentage values for radioactivity remaining in the glands were plotted on semilogarithmic paper against time. The graphed relationship for both species was approximately linear although in the rat values obtained towards the end of the experimental period resulted in a flattening of the curve (Fig. 1). The biological half-life of radioiodine in the thyroid, as calculated from the linear portion of the curve, was found to be approximately 3.5 days in the rat. The half-life value for the guinea pig was much longer, about 26 days. This figure had to be obtained by extrapolation, since the radioactivity remaining in the gland by the end of 3 weeks was too low for accurate measurement.

Discussion. The value of 3.5 days for the biological half-life of thyroidal radioiodine shown here for the rat lies between that of 4.5 days reported by Fish *et al.*(4) and 2.0 days (our calculation) by Taurog and Chaikoff(5) in animals fed a Purina diet, and approximates those obtained in other experiments for which dietary iodine was considered adequate(1,3). In rats with low iodine intake(2,4,6,7) biological half-life values are more variable (2-11 days) with the age of the animal apparently a complicating factor. The rate of disappearance of labelled iodine from the rat thyroid has recently been considered an indication of hormonal secretion rate even though recirculation of radioiodide through the gland introduces some error in the estimation of true hormonal release(1-4). Nevertheless, thyroxine secretory rates so calculated approximate those obtained with the classical goitre prevention method(8). It is evident that the biological half-life of radioiodine in the thyroid of the guinea pig exceeds even the highest estimates given for the rat. The slow rate of biologic decay in the guinea pig thyroid moreover can be accelerated by administration of thyrotrophin or by exposure to cold (unpublished experiments), conditions known to increase hormone release from the gland. Inasmuch as the thyroid I^{127} was not measured in this study no attempt will be made to calculate the daily thyroxine output for the guinea pig. It is highly probable, however, that it is much less than in the rat.

The relatively low rate of hormone secretion as postulated for guinea pig thyroid would be consistent with a number of other observations of thyroid function in this species. The I^{131} uptake per mg of guinea pig thyroid is appreciably less than that of the rat under both *in vivo*(9,10) and *in vitro*(11) conditions. The inactive histological picture of the guinea pig thyroid in contrast to that of the rat is well known. A comparison of conversion ratios (P.B.I. I^{131}/Total I^{131} of plasma) for rats and guinea pigs at 13-16 hours after injection reveals a much higher rate of release of thyroid hormone to the blood in the former species(12). Rates of oxygen consumption, often used as an indicator of the peripheral effects of thyroid hormone, also differ. Herrington(13) finds that for adult guinea pigs and rats at thermal neutrality the heat production is about 590 and 680 calories per square meter per 24 hours respectively. In this laboratory the oxygen consumption per hour per 100 g body weight for the guinea pig is approximately 40% less than that of the rat. TSH concentration in the pituitary and blood of the guinea pig is considerably less than that of the rat(9,14). The difference in the rate of decay of thyroidal radioiodine in the rat and guinea pig is therefore consistent with measurements of other parameters of thyroid-pituitary function in these species.

Summary and conclusions. 1. The rate of biological decay of thyroidal I^{131} was compared in rat and guinea pig by determining the reduction in radioactivity over the gland utilizing the *in vivo* detection technic. The biological half-life of radioiodine was estimated to be 3.5 and 26 days for the rat and guinea pig respectively. 2. The results are considered to suggest that thyroid hormone secretion in the guinea pig normally proceeds at a much slower rate than in the rat. Consideration of other parameters of pituitary-thyroid function for both species supports this view.

1. Wolff, J., *Endocrinology,* 1951, v48, 284.

2. Albert, A., *ibid.,* 1951, v48, 334.

3. Perry, W. F., *ibid.,* 1951, v48, 643.

4. Fish, W. A., Carlin, H., and Hickey, F. C., *ibid.,* 1952, v51, 282.

5. Taurog, A., and Chaikoff, I. L., *J. Biol. Chem.,* 1947, v169, 49.

6. Albert, A., and Tenney, Alida, PROC. SOC. EXP. BIOL. AND MED., 1951, v77, 202.

7. Randall, R. V., Lorenz, Nona, and Albert, A., *Endocrinology,* 1951, v48, 399.

8. Dempsey, E. W., and Astwood, E. B., *ibid.,* 1943, v32, 509.

9. D'Angelo, S. A., Stevens, C. E., Paschkis, K. E., and Cantarow, A., PROC. SOC. EXP. BIOL. AND MED., 1953, v83, 181.

10. D'Angelo, S. A., Stevens, C. E., Paschkis, K. E., Cantarow, A., Sunderman, F. W., and Friedler, G., *Endocrinology,* in press.

11. Rand, C. G., Lee, N. D., and Williams, R. H., PROC. SOC. EXP. BIOL. AND MED., 1951, v78, 307.

12. Chaikoff, I. L., Taurog, A., and Reinhardt, W. O., *Endocrinology,* 1947, v40, 47.

13. Herrington, L. P., *Am. J. Physiol.,* 1940, v129, 123.

14. D'Angelo, S. A., *Endocrinology,* 1953, v52, 331.

Received March 23, 1954. P.S.E.B.M., 1954, v85.

Effect of Corticotropin and Cortisone on Globule Leucocytes of Rat.* (20975)

JOHN F. KENT, BURTON L. BAKER, DWIGHT J. INGLE, AND CHOH HAO LI.
From the Department of Anatomy, University of Michigan Medical School, Ann Arbor; Research Laboratories, The Upjohn Co., Kalamazoo, Mich.; and Hormone Research Laboratory, Department of Biochemistry, University of California, Berkeley.

Globule leucocytes are found frequently in the mucous membranes of the alimentary and lower respiratory passageways of various animals, including the rat. These cells are distinguished by the presence in their cytoplasm of acidophilic metachromatic granules (Fig. 1). Globule leucocytes appear to be normal cells and seldom, except under abnormal conditions, show evidence of degeneration. In this respect they differ from plasma cells which are undergoing transformation to Russell body cells. Globule leucocytes arise by direct transformation of other cells, many origins having been suggested, including eosinophilic leucocytes, wandering cells, plasma cells, mast cells, and lymphocytes. It is only between the small lymphocyte and the globule leucocyte, however, that a complete series of intermediate stages has been described(1).

The functional significance of globule leucocytes is unknown, although their occurrence in the urinary tract of the rat has been correlated with the presence of a nematode infestation(2). Since adrenocortical hormones

---

* Supported (in part) by research grants from the National Institutes of Health and the University of Michigan Memorial-Phoenix Project.

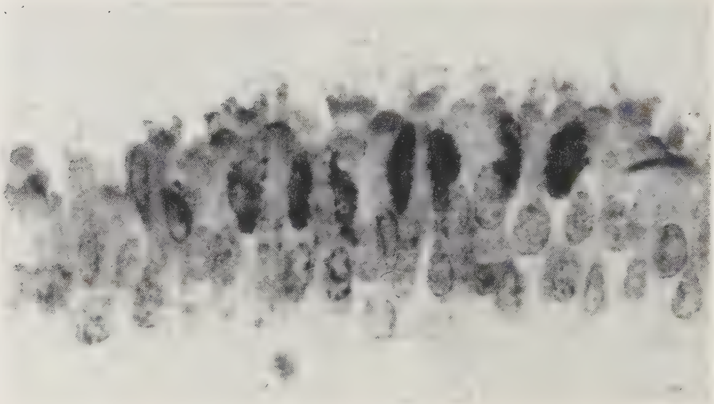


FIG. 1. Several globule leucocytes with their darkly stained granules are present in the tracheal epithelium.

cause a striking reduction in the mass of lymphoid tissue(3,4), in the number of circulating lymphocytes(5,6), and eosinophiles (6,7), information concerning the response of the globule leucocytes to these hormones might throw additional light on the origin and functional significance of these cells.

Procedure. Mature Sprague-Dawley rats of both sexes were used. Food intake was controlled by feeding with a stomach tube or by pair-feeding. Corticotropin was administered by continuous subcutaneous injection, and cortisone acetate[†] by daily injections (Table I). Control animals received physiological saline or Merck Vehicle No. 1 which were used as injection media for the hormones. The adrenalectomized rats were maintained on 1% NaCl solution as drinking fluid and were sacrificed on the 21st postoperative day. Sections of trachea were stained by the Masson trichrome technic or with phosphotungstic acid hematoxylin and basic fuchsin. The number of globule leucocytes in the lining epithelium was counted in 5 well-spaced sections and averaged for each animal.

TABLE I. Effect of Corticotropin and Cortisone on Globule Leucocytes in the Trachea.

Treatment*	No. rats	Daily dose (mg)	Days of treatment	Mean No. glob. leuc./section
C	8	1	21	53 ± 25
S	9	—	21	228 ± 84
C	6	6	21	.2 ± .2
S	5	—	21	111 ± 35
CA	5	5	21	8 ± 2
V 1	5	—	21	242 ± 42
CA	3	10	9	.5 ± .3
V 1	2	—	9	168 ± 30
CA	1	10	14	.4 ± .4
V 1	1	—	14	193 ± 18

* C = corticotropin; S = saline; CA = cortisone acetate; V = vehicle.

Results. The administration of corticotropin or cortisone acetate caused a sharp reduction in the number of globule leucocytes present in the tracheal mucosa (Table I). At the higher dose levels, these cells were eliminated almost completely from the mucous membrane. Adrenalectomy, on the other hand, exerted no consistent effect on the number of globule leucocytes in the trachea or colon in the 36 animals studied. With respect to the types of cells from which globule leucocytes are believed to arise, only the eosinophilic leucocyte and the lymphocyte exhibit such great sensitivity to corticotropin and cortisone. This sensitivity of the globule leucocyte to the action of adrenocortical hormones tends to support the anatomical evidence(1) previously cited which indicates that the globule leucocyte arises by transformation of lymphocytes. The elimination of globule leucocytes from the body by hormonal treatment provides a valuable tool for use in experiments designed to analyze the factors which are responsible for the appearance of these cells. Also, the question must be raised of the possible participation of these cells in defense against respiratory infection since the creation of a state of hyperadrenocorticalism in rats increases the frequency of respiratory disease.

[†] We wish to thank Dr. Elmer Alpert of Merck and Co., Inc., for the cortisone acetate and Vehicle No. 1.

Summary. Treatment for 21 days with one mg of corticotropin or 5 mg of cortisone acetate daily caused a marked decline in the population of globule leucocytes in the tracheal mucosa of rats. Administration of 6 mg corticotropin or 10 mg cortisone acetate daily caused these cells to disappear almost completely. In this response, the globule leucocyte resembles the lymphocyte.

1. Kent, J. F., *Anat. Rec.*, 1952, v112, 91.
2. Kirkman, H., *Am. J. Anat.*, 1950, v86, 91.
3. Baker, B. L., Ingle, D. J., and Li, C. H., *ibid.*, 1951, v88, 313.
4. Dougherty, T. F., and White, A., *Am. J. Anat.*, 1945, v77, 81.
5. ———, *Endocr.*, 1944, v35, 1.
6. Forsham, P. H., Thorn, G. W., Prunty, F. T. G., and Hills, A. G., *J. Clin. Endocr.*, 1948, v8, 15.
7. Gordon, A. S., Piliero, S. J., Landau, D., *Endocr.* 1951, v49, 497.

Received March 23, 1954. P.S.E.B.M., 1954, v85.

Effect of Epinephrine on Thyroid Hormone Level in Blood. (20976)

C. D. ESKELSON, H. E. FIRSCHEIN, AND H. JENSEN.
From the Department of Biochemistry, Army Medical Research Laboratory, Fort Knox, Ky.

Thyroid functional activity, employing radioiodide (I^{131}), has been related to the concentration of organic-bound I^{131} in blood. The level of organic-bound iodine in blood is generally regarded as an accurate index of the circulating thyroid hormone.* The concentration of organic iodine in blood represents the difference between the amount of hormone secreted by the thyroid and the amount of hormone metabolized or excreted during the same interval of time. Williams, Jaffe and Kemp(2) found that epinephrine administration to rats produced a lowering of I^{131} concentration in the thyroid and in the organic-bound I^{131} in the blood. Soffer and his associates(3) observed that the parenteral injection of epinephrine into both the intact and the totally thyroidectomized dog resulted in an increase in secretion of thyrotropin (TSH) from the anterior pituitary. Botkin and Jensen(4) reported that while both TSH and epinephrine administration to rats produced a lowering of the I^{131} concentration in the thyroid gland, the organic-bound I^{131} in the blood was increased after TSH injection but decreased after epinephrine administration. This latter response was interpreted as being due to an increased utilization of circulating thyroid hormone caused by the epinephrine.

In order to study further the influence of epinephrine on blood organic iodine the effect of simultaneous administration of TSH and epinephrine or of thyroxin and epinephrine on the level of organic I^{131} in blood was investigated.

Methods and materials. Male rats of the Sprague-Dawley strain, weighing from 200 to 250 g, were used in all experiments. The animals were housed at 25°C ± 1° and were fed Purina checkered dog chow. Food, but not water, was withheld for 18 to 20 hrs before sacrifice. Eighteen hrs before sacrifice the animals receiving TSH† (dissolved in 0.9% saline) were injected with 5 μc I^{131},‡ and those receiving thyroxin (dissolved in NaOH at

---

* Recently Gross and Pitt-Rivers(1) have presented evidence that the thyroid releases, in addition to thyroxin, another active principle which was shown to be 3:5:3′-triiodothyronine.

† TSH used was obtained from two sources: A Parke, Davis preparation containing 10 J. S. units/mg, and an Armour preparation containing 5 J. S. units/mg. The authors greatly appreciate the generosity of Parke, Davis and Co., Detroit, Mich., and of Armour and Co., Chicago, Ill., in supplying these preparations.

TABLE I. Effect of Simultaneous Administration of TSH and Epinephrine on Organic-Bound I^{131} in Blood.

Treatment	No. of animals	OBI, C/min.	Rat No.	Statistical significance
Exp. 1				
TSH + epinephrine	6	56 ± 18*	1- 6	$p < .01$ $f = 15.96$
TSH	6	68 ± 21	7-12	
Epinephrine	6	24 ± 10	13-18	
Saline	6	37 ± 11	19-24	
Exp. 2				
TSH + epinephrine	4	74 ± 7	1- 6†	$p < .01$ $f = 15.96$
TSH	6	84 ± 17	7-12	
Epinephrine	6	32 ± 3	13-18	
Saline	6	37 ± 10	19-24	

* Mean ± stand. dev.

† 2 samples lost.

All animals received 5 μc I^{131} 18 hr before sacrifice.

No. 1-12 were inj. with 24 J.S. units TSH in 1 ml 0.9% saline 2½ hr before sacrifice.

No. 1-6 and 13-18 were inj. with 0.1 mg epinephrine in 1 ml of 0.9% saline 2 hr before sacrifice.

No. 19-24 were inj. with 1 ml 0.9% saline 2 hr before sacrifice.

pH 11.0) were injected with 30 μc of I^{131}. All injections were given intraperitoneally. Further details as to the amount and time of TSH, thyroxin and epinephrine§ injections are given in the various tables. The animals were sacrificed by ether anesthesia and blood was drawn by heart puncture. The blood was allowed to clot and the serum was obtained by centrifugation. The organic-bound I^{131} content in serum was determined according to the procedure described by Botkin and Jensen (4).

Results. As can be seen from the data of Table I, the increased organic I^{131} blood level, observed after TSH injection alone, became lowered on simultaneous administration of TSH and epinephrine. The highest to lowest organic I^{131} level in blood respectively was observed with TSH only, TSH and epinephrine, saline control, and lowest with epinephrine alone.

The data of Table II indicate that there was no difference in the response of the gland with regard to the I^{131} distribution whether TSH was administered alone or in combination with epinephrine.

The data of Table III illustrate that the highest organic I^{131} values in blood were observed in the saline control group and the lowest values in the thyroxin-epinephrine group. It is interesting to note that thyroxin alone exerted a greater effect on lowering the organic I^{131} blood level than did epinephrine alone. The highest to lowest organic I^{131} level in blood respectively was found with saline, epinephrine, thyroxin, and lowest with thyroxin-epinephrine.

Discussion. The data presented indicate that epinephrine administration produced a lowering of the organic-bound I^{131} in blood. This response was observed on administration of epinephrine alone as well as in combination with either TSH or thyroxin. These findings support the postulation of Botkin and Jensen (4) that epinephrine produced an increased utilization of circulating thyroid hormone by the peripheral tissues.

The estimation of the blood organic I^{131} level is, therefore, inadequate in itself as a test of thyroid function. It merely measures the amount of circulating thyroid hormone at a certain time period.

Summary. Administration of epinephrine alone or in combination with either TSH or

TABLE II. Effect of Simultaneous Administration of TSH and Epinephrine on I^{131} Distribution in the Thyroid.* Four animals in each series.

Treatment	Rat No.	Gland Total Cpm × 10^3	Gland Organic Cpm × 10^3
		Mean	Mean
TSH + epinephrine	1- 4	16.30	10.95
TSH	5- 8	16.25	11.35
Epinephrine	9-12	22.19	15.96
Saline	13-16	25.87	20.92

* Distribution of I^{131} was determined according to the method of Morton and Chaikoff (*J. Biol. Chem.*, 1943, v147, 1).

All animals inj. with 5 μc I^{131} 18 hr before sacrifice.

No. 1-8 received 2.4 mg TSH (24 J.S. units) 2 hr before sacrifice.

No. 1-4 and 9-12 received 0.1 mg epinephrine 1½ hr before sacrifice. No. 13-16 received saline 2 hr before sacrifice.

---

‡ The radioactive iodide, I^{131}, was supplied by the Oak Ridge National Laboratory on allocation from the Isotope Division, U. S. Atomic Energy Commission.

§ Soluble Salt, The Upjohn Co., dissolved in saline.

TABLE III. Effect of Simultaneous Administration of Thyroxin and Epinephrine on Organic-Bound I^{131} in the Blood.

Treatment	No. of animals	OBI, C/min.	Rat No.	Statistical significance
Exp. 1				
Thyroxin + epinephrine	5	91.8 ± 30.2*	1- 5	$f = 38.91$
Thyroxin	5	94.8 ± 28.2	6-10	$p < .01$
Epinephrine	5	132.0 ± 18.5	11-15	
Saline	3	152.0 ± 25.7	16-18	
Exp. 2				
Thyroxin + epinephrine	5	117.8 ± 10.6	1- 5	$f = 9.37$
Thyroxin	5	186.0 ± 46.5	6-10	$p < .01$
Epinephrine	5	196.0 ± 65.8	11-15	
Saline	3	299.7 ± 17.7	16-18	
Exp. 3				
Thyroxin + epinephrine	5	189.0 ± 33.0	1- 5	
Thyroxin	5	242.0 ± 82.0	6-10	
Epinephrine	5	246.0 ± 64.0	11-15	
Saline	1	318.0	16-18†	

* Mean ± stand. dev. † 2 samples lost.

Each animal inj. with 30 μc I^{131} 18 hr before sacrifice.

No. 1-10 inj. with 0.5 mg of thyroxin 2½ hr before sacrifice. No. 1-5 and 11-15 inj. with 0.1 mg epinephrine 2 hr before sacrifice. No. 16-18 inj. with a normal saline solution 2 hr before sacrifice.

thyroxin has been found to produce a lowering in the blood level of thyroid hormone (organic-bound I^{131}). This response to epinephrine is interpreted as being due to the increased utilization of circulating thyroid hormone caused by epinephrine. The significance of this finding is discussed.

The authors are indebted to Dr. G. L. Saiger for the statistical analysis of the data.

1. Gross, J., and Pitt-Rivers, R., *Biochem. J.*, 1953, v53, 645.

2. Williams, R. H., Jaffe, H., and Kemp, C., *Am. J. Physiol.*, 1949, v159, 291.

3. Soffer, L. J., Volterra, M., Gabrilove, J. L., Pollack, A., and Jacobs, M., Proc. Soc. Exp. Biol. and Med., 1947, v64, 446.

4. Botkin, A. L., and Jensen, H., *Endocrinology*, 1952, v50, 68.

Received March 29, 1954. P.S.E.B.M., 1954, v85.

The First Heart Sound in Ventricular Contractions Arising from the Apex and Base.* (20977)

Roy D. Schaefer† and Robert C. Little.

From the Divisions of Physiology and Medicine, University of Tennessee, Memphis.

The first heart sound has been reported to be delayed in ectopic ventricular beats in humans(1,2) and in dogs(2). This delay was attributed to the mechanism of closure of the A-V valves. No attempt was made in these reports to differentiate between ectopic beats arising from the apex and those originating from the base. The mode of contraction may be different in beats arising from one or the other of these locations; therefore, variations in closure of the A-V valves and timing of the first sound might occur. The present study was undertaken to investigate this pos-

---

* Based on a thesis presented by Roy D. Schaefer in partial fulfillment of the requirements for the degree of M. S. in Physiology. This study supported by a grant from the Am. Heart Assn. and the Shamokin-Coal Township Heart Assn.

† Public Health Service Research Fellow of the National Heart Institute.

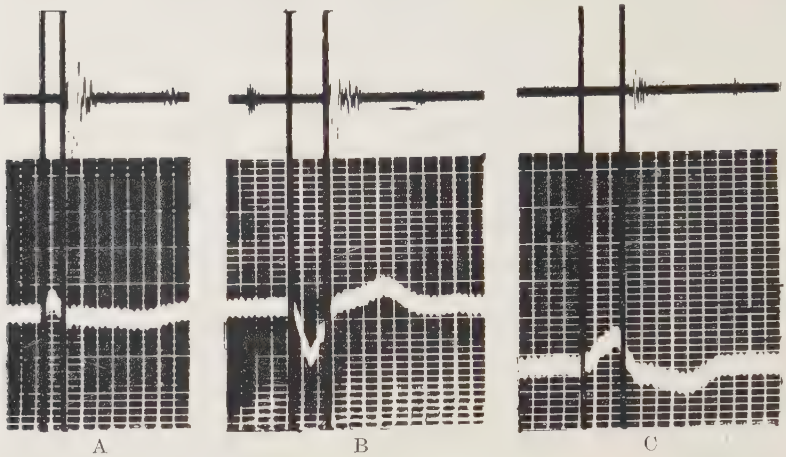


FIG. 1. Representative examples of a normal beat (A), an ectopic beat from the apex (B), and an ectopic beat from the base (C). Each is taken from the record of Patient III.

sibility and to explore further the effect of ventricular contractions on the timing of the first heart sound.

Method. Heart sounds were recorded from the exposed hearts of anesthetized dogs by the method of Little, *et al.*(2). Lead II of the electrocardiogram was simultaneously recorded with the sound tracing at a camera speed of 50 mm per second. No parallax existed between recording beams. Two small fish-hook electrodes were thrust into the myocardium one cm apart near the apex of the heart and 2 were similarly placed at the base of the pulmonary conus. Each pair of electrodes was connected to a Harvard inductorium. The heart was stopped by stimulation of the right vagus by means of a tetanizing current from a third inductorium. While the heart was stopped, single make and break induction shocks were used to produce ectopic ventricular contractions from the base or from the apex. Records of human cases of ectopic ventricular systoles were recorded with a Cambridge phonocardiograph at a camera speed of 50 mm per second. Lead II of the electrocardiogram was simultaneously recorded.

Results. Records from 5 dogs and 5 human subjects were suitable for analysis.‡ The sound records were free from artifacts, and the beginning of the first sound was not obscured by auricular sounds. The time interval between beginning of the QRS deflection and onset of the first sound was measured, by means of adequate magnification, to 0.005 second. This interval has been termed the "heart sound time"(2).

In ectopic ventricular beats, the QRS complex is broad, slurred, and notched, and the T wave extends in the opposite direction from the principal deflection of the QRS. In beats arising from an ectopic focus in the ventricular base the major deflection of the QRS complex is upright in all 3 standard electrocardiographic leads. If the beat originates in the apex the major deflection of the QRS is down in all 3 standard leads. Representative examples of a normal beat, an ectopic contraction from the base, and an ectopic contraction from the apex are shown in Fig. 1.

The mean heart sound times in dogs for normal beats, ectopic beats from the base, and ectopic beats from the apex are presented in Table I. In each animal the heart sound time for apical ectopic beats is longer than for normal beats, and the interval for ectopic contractions from the base is longer than for beats from the apex. The average of the means, or grand mean, is 0.048 second for the normal beats, 0.080 for the apical beats, and 0.093 second for beats from the base. Similar data from the human cases are presented in Table II. Only 2 of these patients had premature beats from both the base and

---

‡ Other observations on records of human subjects have been previously reported(2).

TABLE I. Mean Heart Sound Times in Dogs.

Dog	Origin of beats	No. of beats measured	Mean time in seconds	Stand. dev. (±)
I	Base	29	.087	.01769
	Apex	23	.077	.00985
	Normal	25	.044	.00878
II	Base	29	.105	.01020
	Apex	30	.089	.00894
	Normal	25	.055	.00877
III	Base	30	.092	.00548
	Apex	27	.078	.00854
	Normal	25	.043	.00480
IV	Base	22	.092	.01086
	Apex	29	.078	.01114
	Normal	25	.055	.00831
V	Base	29	.090	.00678
	Apex	30	.077	.00520
	Normal	25	.044	.00794

the apex. In these 2 cases the first heart sound was delayed in apical premature beats as compared with the normals, and was delayed to a greater extent in basal premature beats. In 2 patients with premature beats only from the apex, the mean heart sound times were longer in the case of the premature than in the normal beats. In the patient with premature beats arising only from the base, the mean heart sound time was markedly longer for the premature beats than for the normal beats. In the human cases the grand mean for the normal beats was 0.040 sec., that for apical premature beats was 0.088 sec., while the grand mean of the basal premature beats was 0.121 sec.

TABLE II. Mean Heart Sound Times in Human Subjects.

Patient	Origin of beats	No. of beats measured	Mean time in seconds	Stand. dev. (±)
I	Base	26	.136	.00866
	Normal	26	.044	.00300
II	Base	26	.128	.01456
	Apex	4	.106	.01109
	Normal	26	.039	.00469
III	Base	21	.099	.01510
	Apex	6	.083	.01536
	Normal	23	.056	.00906
IV	Apex	25	.073	.00985
	Normal	25	.029	.00574
V	Apex	16	.088	.01661
	Normal	16	.031	.00469

The probability of the occurrence of the observed time differences by chance alone in the dog experiments was computed by means of the T test, for normal beats as against apical ectopic beats, for normal beats as against basal ectopic beats, and for apical ectopic beats as compared with basal. These tests indicate that the time differences between apex and base in Dog 1 might be expected to occur by chance alone in 2 to 5% of similar experiments. For all of the remaining comparisons in all animals, the probability level is less than 0.1%.

A similar statistical analysis was carried out on the data obtained from the human records. This shows that the observed time differences between base and apex in Patient II would be expected to occur by chance alone in from 0.1 to 1% of a similar group of measurements. In Patient III the observed differences between ectopic beats initiated in base and apex might occur by chance alone in over 5% of a similar series of measurements. But in all of the other comparisons in patients the probability level is less than one in a thousand that chance alone would account for the measured time differences.

The method of sound recording used in this work was not adequate for accurate measurement of the intensity of the heart sounds. However, in many ectopic systoles the sound was of less intensity than that of normal beats. This would tend to refute the implication of Cossio *et al.*(1) that only the intensified extrasystolic first sounds are delayed. Our data indicate that the delay in the first sound occurs in all ectopic beats, regardless of the intensity of the sound.

Discussion. This study confirmed the observations(1,2) that the heart sound time is prolonged in ectopic ventricular contractions. Previous studies(2) on animals employed ventricular pressure curves to indicate the onset of ventricular contraction. This investigation utilized the electrocardiogram to indicate the onset of ventricular depolarization. As a result of the time difference between mechanical and electrical events, the heart sound times reported in animals are slightly longer than those found by previous authors. The relative increase in heart sound time in ectopic beats is, however, of the same magnitude.

The reason for the greater delay of the first sound in basal as compared to apical ectopic beats remains speculative. When the ectopic focus is in the base of the ventricle, the impulse probably must proceed through the ventricular wall toward the apex and then through the wall of the other ventricle. However, if the ectopic focus is at the apex, it could travel simultaneously through both ventricular walls. In other words, the wave of depolarization would require a longer time to activate sufficient ventricular muscle mass to inaugurate contraction in basal ectopic beats. When auricular systole is absent the valves must be closed from the open position by ventricular contraction(3). The greater delay in the beginning of ventricular contraction in basal ectopic beats could thus explain the greater delay in onset of the first sound.

Summary. 1. Simultaneous electrocardiograms and phonocardiograms were recorded from 5 human subjects having ectopic ventricular systoles. Similar tracings were obtained from 5 dogs in which ectopic systoles were produced by induction shocks to the exposed heart during vagal arrest. The time interval between beginning of ventricular depolarization and onset of the first heart sound was measured for normal and for ectopic beats. The data were subjected to statistical analysis. 2. The beginning of the first heart sound was significantly delayed in ectopic ventricular contractions. The first sound occurred significantly later in ectopic beats arising from the ventricular base than in those from the apex. The probable reasons for these findings are discussed.

---

1. Cossio, P., Dambrosi, R. G., and Warnford-Thomson, H., *Brit. Heart J.*, 1947, v9, 275.

2. Little, R. C., Hilton, J. G., and Schaefer, R. D., *Circ. Research*, 1954, v2, 48.

3. Little, R. C., *Am. J. Physiol.*, 1951, v166, 289.

Received March 29, 1954. P.S.E.B.M., 1954, v85.

Toxicity of Choline and Dimethylethanolamine in the Guinea Pig and the Rat.* (20978)

W. E. CORNATZER.

From the Department of Biochemistry, University of N. Dakota Medical School, Grand Forks, N. D.

The toxicity of choline in the rat has been established(1-3). The metabolism of choline in the liver is related to the activity of choline oxidase(4,5). This enzyme system has a very low activity or is entirely lacking in the liver of the guinea pig and thus choline deficiency cannot be produced by dietary means (6). Dimethylethanolamine, a precursor of choline(7), is not oxidized by liver choline oxidase(8). In view of these observations it was of interest to determine if there was a difference in the toxicity of choline or dimethylethanolamine in the guinea pig and rat.

Methods. Male albino rats (100-110 g) and guinea pigs (200-300 g) were divided into two groups. Group 1 received intraperitoneally 45 mg of dimethylethanolamine or choline (base) per 100 g of body weight in 1 cc and Group 2 received 60 mg. Animals which lived longer than 30 minutes in most cases survived.

Results. From the results in Table I it was apparent that there was no difference in the toxicity of choline in the guinea pig or

TABLE I. Toxicity of Choline and Dimethylethanolamine in the Guinea Pig and Rat.

mg (base) /100 g of body wt		Rats		Guinea pigs	
		No. of animals	% mortality	No. of animals	% mortality
45	Choline	14	29	45	20
	DME*	24	75	28	71
60	Choline	20	60	39	74
	DME	25	88	22	77

* Dimethylethanolamine.

---

* This investigation supported (in part) by a research grant from the Division of Research Grants and Fellowships of the National Institutes of Health, U. S. Public Health Service.

rat. The absence of choline oxidase in the liver of the guinea pig does not increase the toxicity of choline. Dimethylethanolamine, which is not metabolized by choline oxidase in the liver, appears to be more toxic than choline in both animals. Dimethylethanolamine is metabolized much slower than choline, for it can be detected in the tissues of rats following the administration of large quantities(8). On the other hand, after similar doses of choline were given, neither free nor combined dimethylethanolamine could be detected in the tissues of the rat. This difference in metabolism of dimethylethanolamine may help account for its increased toxicity.

Summary. 1. The toxicity of choline and dimethylethanolamine in the guinea pig and the rat has been compared. 2. There is very little difference in the toxicity of choline or dimethylethanolamine in the guinea pigs and rats. However, dimethylethanolamine appears to be the more toxic.

---

1. Hodge, H. C., and Goldstein, M. R., PROC. SOC. EXP. BIOL. AND MED., 1942, v51, 281.

2. Hodge, H. C., *ibid.*, 1944, v57, 26.

3. Neuman, M. W., and Hodge, H. C., *ibid.*, 1945, v58, 87.

4. Handler, P., and Bernheim, F., *J. Biol. Chem.*, 1942, v144, 401.

5. Humoller, F. L., and Zimmerman, H. J., *Am. J. Phys.*, 1953, v174, 199.

6. Bernheim, F., and Bernheim, M. L. C., *Am. J. Phys.*, 1938, v121, 55; Handler, P., PROC. SOC. EXP. BIOL. AND MED., 1949, v70, 70.

7. Du Vigneaud, V., Chandler, J. P., Simmonds, S., Moyer, A. W., and Cohn, M., *J. Biol. Chem.*, 1946, v164, 603; Artom, C., and Cornatzer, W. E., *ibid.*, 1948, v176, 949.

8. Artom, C., in McElroy, W. D., and Glass, B., *Phosphorus Metabolism*, v11, 203, Johns Hopkins Press, 1952.

Received March 29, 1954. P.S.E.B.M., 1954, v85.

Effect of ACTH on Distribution of Plasma Proteins of Patients with Pulmonary Tuberculosis. (20979)

L. C. SMITH, E. J. DESAUTELS, AND GLADYS J. DOWNEY.
(Introduced by Smith Freeman.)

From the Research Division and the Tuberculosis Service, Veterans Administration Hospital, Hines, Ill.

Vaughan and coworkers(1) have shown that both ACTH and cortisone therapy depress fibrinogen and gamma globulin levels in the blood of patients with active rheumatoid arthritis and scleroderma. Reiner(2) likewise has shown that these agents increase albumin concentration, but decrease gamma globulin in sera of patients with *disseminated lupus erythematosus.* Studies reported in this communication describe the changes which occur in the electrophoretic pattern of the plasma proteins of tuberculous patients following administration of ACTH. The results will be shown to be similar to that described in(1,2).

Material and methods. Clinical material. Heparinized plasma samples were obtained from 11 patients hospitalized for pulmonary tuberculosis. These samples were generally obtained before, at 2-week intervals during, and after treatment with ACTH. ACTH was generally administered in divided doses intramuscularly each 6 hours. Seventy-five to 100 mg were generally given at onset of treatment, and then the amount was gradually tapered to 20 mg daily before being discontinued.

Electrophoretic fractionation. Diagrams were obtained under nearly the same experimental conditions in order to make comparisons possible. The plasma was diluted 1-6 with veronal buffer solution, pH 8.6, ionic strength 0.1; electrophoresis was conducted in the standard 11 ml cells of the Klett apparatus. Ten milliamperes of current were passed until separations were satisfactory; this was generally about 3 hours. Enlarged tracings were made of anode pattern photographs and per cent of total plasma proteins deter-

TABLE I. Electrophoretic Distribution of Serum Proteins in Normal and Pulmonary Tuberculosis Patients.

Avg and probable error of mean result	Total protein (g %)	A/G ratio	Relative % of total proteins					
			Albumin	Alpha 1	Alpha 2	Beta	Gamma	Fibrinogen
For 12 normal men	6.94±.05	1.38	5.40±.6	4.6±.19	12.4 ±.62	9.8±.56	12.5± .63	6.6±.19
For 11 untreated tuberculosis patients	7.00±.17	.62	32.7 ±.84	8.6±.39	13.00±.45	12.3±.49	20.3±1.01	13.9±.37

mined(3). Total proteins were determined by the conventional Kjeldahl method using a nitrogen factor of 6.25.

Results. Table I presents the average values obtained on the 11 patients before treatment as compared to those of 12 normal adults. The values obtained are similar to those reported by Seibert *et al.*(4) for tuberculous patients. The albumin value and A/G ratios of the tuberculous patients are definitely less than normal and there is an increase in gamma globulin. In addition, fibrinogen and alpha 1 globulin values of the patients with active tuberculosis are approximately double those of the normal individuals. Our results differ from those of Seibert *et al.*(4) in that we were unable to demonstrate a significant increase in the alpha 2 globulin.

Table II shows the values obtained on the 11 patients during hormone treatment. The average increase in albumin was from 32.7% to 40.8%. The average fall in fibrinogen was from 13.9% to 9.8%. Gamma globulin likewise fell from 20.3% to 14.3%. The values for the alpha 2, and beta globulins, although not distinctly abnormal from the beginning, appeared to be variable. Although the alpha 1 globulin level was initially considerably higher, the changes brought about by therapy were not consistent so that the net average change was small.

Plasma of patients receiving large, prolonged dosages of ACTH sometimes showed rather rapid changes in certain of the components. Table III illustrates the changes obtained with patient F. W. during a course of therapy. It may be noted that within one week after the start of therapy, the albumin increased from 34.9% to 41.3%, while fibrinogen decreased from 13.2% to 8.4%. On the other hand the gamma globulin and total protein did not change appreciably. This is in agreement with Vaughan *et al.*(1): fibrinogen levels are more readily affected by treatment than gamma globulin levels. It is also evident that reduction or cessation of ACTH treatment resulted in reversion of the protein fractions to values similar to those noted in the control samples. Le Maistre *et al.*(5) have likewise shown that cessation of hormone administration caused gamma globulin values to rise to initial values by the end of 4 weeks.

TABLE II. Effect of ACTH on Albumin, Fibrinogen, and Gamma Globulin of 11 Pulmonary Tuberculosis Patients.

	Albumin			Fibrinogen			Gamma globulin		
	Control	Peak	Change	Control	Peak	Change	Control	Peak	Change
	29.2	44.7	+15.5	16.8	10.3	−6.5	15.8	10.2	− 5.6
	30.9	43.8	+12.9	13.1	8.2	−4.9	22.8	9.0	−13.8
	32.6	37.8	+ 5.2	14.2	11.6	−2.6	25.2	16.8	− 8.4
	25.9	27.7	+ 1.8	15.2	15.0	− .2	19.8	20.3	+ .5
	33.8	39.1	+ 5.3	10.0	9.1	− .9	29.2	24.5	− 4.7
	39.2	40.8	+ 1.6	15.7	8.9	−6.8	11.9	7.8	− 4.1
	34.0	42.6	+ 8.6	12.6	10.0	−2.6	20.2	8.2	−12.0
	27.4	30.7	+ 3.3	14.3	12.4	−1.9	20.6	21.6	+1.0
	38.8	48.6	+ 9.8	13.0	6.6	−6.4	17.0	10.3	− 6.7
	33.2	45.3	+12.1	14.6	8.2	−6.4	24.4	15.7	− 8.7
	34.9	47.2	+12.3	13.2	8.0	−5.2	16.2	12.6	− 3.6
Avg	32.7	40.8	+ 8.04	13.9	9.8	−4.0	20.3	14.3	− 6.3

TABLE III. Electrophoretic Distribution of Plasma Proteins in Pulmonary Tuberculosis before and after ACTH Therapy. (Last patient, Table II.)

Date	ACTH admin., mg/day	Albumin, %	Fibrinogen, %	Gamma globulin, %	A/G ratio	Total protein, g %
8– 8–49	Control	34.9	13.2	16.2	.67	6.72
9	100					
11						
12	75					
15		41.3	8.4	16.0	.82	6.65
18						
19	100					
25		43.2	9.4	14.2	.91	5.86
9–12		47.2	8.0	12.6	1.05	6.62
15						
16	75					
22						
23	25					
24	0	35.4	14.6	13.6	.86	5.91
26						

Summary. 1. Albumin level and A/G ratio of pulmonary tuberculosis patients are reduced from normal value. 2. Levels of gamma globulin, fibrinogen, and alpha 1 globulin are considerably higher than those of normal individuals. 3. ACTH depresses the gamma globulin and fibrinogen, and raises the albumin values and A/G ratio toward normal levels. Gamma globulin is less susceptible to immediate changes than the latter two components. 4. Cessation or reduction of ACTH therapy brought about a reversion of protein fractions to values similar to those noted in control samples. 5. Values for alpha 1, alpha 2, and beta globulins appeared to be variable under the influence of ACTH.

1. Vaughan, J. H., Bayles, T. B., and Favour, C. B., PROC. SOC. EXP. BIOL. AND MED., 1951, v76, 274.

2. Reiner, M., *ibid.,* 1950, v74, 529.

3. Tiselius, A., and Kabat, E. A., *J. Exp. Med.,* 1939, v69, 119.

4. Seibert, F. B., Seibert, M. V., Atno, A. J., and Campbell, H. W., *J. Clin. Invest.,* 1947, v26, 90.

5. Le Maistre, C. A., Tompsett, R., Muschenheim, C., Moore, J. A., and McDermott, W., *ibid.,* 1951, v30, 445.

Received March 29, 1954. P.S.E.B.M., 1954, v85.

Effect of Dietary *Clostridia* upon Growth-Promoting Responses of Penicillin.* (20980)

J. F. ELAM, R. L. JACOBS, JEAN FOWLER, AND J. R. COUCH.

From the Departments of Poultry Husbandry and Biochemistry and Nutrition, Texas Agric. Exper. Station, College Station, Texas.

Elam *et al.*(1) reported that penicillin greatly reduced the total number of *Clostridia* present in the intestinal tract of the chick and that small amounts of the antibiotic inhibited the growth of *Clostridia* isolates *in vitro.* Jacobs *et al.*(2) later reported that antibiotics (directly or indirectly) stimulated growth by reducing the total number of *Clostridia* in the intestinal tract of the chick and that the antibiotics may be ineffective in stimulating growth in a clean environment where the *Clostridia* population was low. Kiser *et al.* (3) reported that a number of *Clostridia* were

---

* This work was supported in part by a grant-in-aid from Merck and Co., Rahway, N. J.

destroyed, in *in vitro* studies, by the addition of aureomycin, penicillin or terramycin. The growth-promoting effect of penicillin and terramycin has been explained as in inhibition of *Clostridium perfringens* in the intestinal tract of turkeys by Sieburth *et al.*(4). Elam *et al.* (5) observed that when meat scraps were added to the basal diet of swine the total number of *Clostridia* was increased and this number was greatly lowered by the addition of chloromycetin which resulted in an increase in growth, whereas the addition of chloromycetin to the basal diet not containing meat scraps (where the total number of *Clostridia* was low) failed to stimulate growth. Coates *et al.* (6,7) reported that chicks reared in quarters not previously used for chick experiments failed to show a growth response to penicillin, whereas a positive response occurred in old quarters previously used for chick experiments. These authors concluded that the reason for the antibiotic response in the old quarters was the presence of an "infection" that made the chicks sensitive to the antibiotic. Similar studies have been reported by others (8-10).

The two experiments described in this paper were conducted to determine the effect of feeding fecal *Clostridia* to the chick in old and clean environment with and without an antibiotic.

Methods. Data from 2 separate experiments are reported in the present study. In each experiment parallel tests were carried out in old and clean quarters. The New Hampshire chicks used in these studies were obtained from hens that had been fed the diet described earlier by Elam *et al.*(11). For the test in the clean environment the birds were housed in a clean room which had been washed daily for a period of 2 weeks before the beginning of the experiments. The room was washed daily during the entire experimental period. In the test carried out in old quarters the birds were housed in quarters which had been used continuously for the past 3 years for chick experiments. The birds were maintained in batteries with raised screen floors. Feed and water were supplied *ad libitum* and the chicks were weighed at weekly intervals during an experimental period of 10 weeks. For the first experiment 350 day-old New Hampshire chicks were randomized into 7 groups of 50 chicks each, and in the second experiment 320 chicks were randomized into 8 groups of 40 chicks each. The *basal diet* used in this study was the all-vegetable protein diet described earlier by Jacobs *et al.*(2). Penicillin was administered at a level of 2 mg per lb of feed. Fresh *fecal material* containing approximately 15,000 *Clostridia* per gram of feces was put in a water solution and mixed directly into the feed every other day at a level of 8 ml (which contained 0.350 g of fecal material per ml) per pound of feed. The water solution of fecal *Clostridia* was prepared by suspending 0.350 g of fecal material per ml of water. The water suspension was then heat shocked for 30 minutes at 65-70°C to kill the aerobic microorganisms. The autoclaved fecal material was prepared by autoclaving the water suspension for 15 minutes at 15 lb of pressure at 121-125°C, and then fed at the same level as the fecal *Clostridia*. Fecal samples were collected once a week and were prepared as described by Elam, Gee and Couch(12). The total number of *Clostridia* per gram of feces was determined by the method described by Jacobs *et al.*(2). An analysis of variance for the *Clostridia* counts and chick weights was made, and statistical significance was determined from the F value (ratio of appropriate mean square values from an analysis of variance table).

Results. In the first experiment the addition of penicillin produced a significant increase in the growth of the chicks and decreased the total number of *Clostridia* per gram of feces when maintained in old quarters (Table I). This was not the case when penicillin was added to the diet of chicks maintained in clean quarters. Under the latter conditions the addition of penicillin was ineffective in increasing growth or reducing the total number of *Clostridia*. This is in agreement with the work reported earlier by Jacobs *et al.*(2). There is also a significant difference between the two control groups as the birds reared in the clean quarters grew more rapidly and had a much lower *Clostridia* count regardless of the antibiotic supplement.

The addition of fecal *Clostridia* to the basal

TABLE I. Effect of Feeding Penicillin and Fecal *Clostridia* upon Weights and Number of *Clostridia* in Old and Clean Quarters.

Group	Avg wt at 10 wk (g)	Total No. of Clostridium isolates (per g of feces)
First experiment		
Old quarters		
Control	969	15570
Penicillin	1085*	265*
Clean quarters		
Control	1167	1215
Penicillin	1210	615
Fecal *Clostridia*	1042*	10260*
" " + penicillin	1180	450
Autoclaved fecal *Clostridia*	1150	1110
Second experiment		
Old quarters		
Control	1134	12450
Penicillin	1262*	640*
Fecal *Clostridia*	1170	16710
" " + penicillin	1310*	740*
Clean quarters		
Control	1324	940
Penicillin	1343	650
Fecal *Clostridia*	1210*	9440*
" " + penicillin	1339	625

* Significant at the 1% level.

diet of birds reared under clean conditions significantly reduced the growth of the birds and increased the total number of *Clostridia* (Table I). When penicillin and fecal *Clostridia* were fed in combination, the growth of the birds was not retarded nor was the total number of *Clostridia* increased. The addition of autoclaved fecal *Clostridia* had no effect on the growth or the *Clostridia* count. The latter was administered to determine whether or not the solution of fecal material would inhibit growth in the absence of *Clostridia*.

In the second experiment the addition of penicillin increased growth and decreased the total number of *Clostridia* in old quarters but failed to have any effect on the growth of the birds or on the fecal *Clostridia* count under clean conditions where the *Clostridia* count was low (Table I). The addition of fecal *Clostridia* failed to have any effect on growth or *Clostridia* count in old quarters as the *Clostridia* population was already high enough to retard growth. As in the first experiment the feeding of fecal *Clostridia* under clean conditions decreased the growth of the birds and increased the *Clostridia* count. The incorporation of penicillin corrected this growth retardation and decreased the *Clostridia* count (Table I).

It should be pointed out that in comparing the old and clean quarters that there is a considerable difference between the *Clostridia* population in the control groups which explains in part the difference in weights between the two quarters. This also explains the response obtained from the antibiotics in contaminated but not in the clean quarters.

Summary. 1. The feeding of penicillin stimulated growth and decreased the *Clostridia* count per gram of feces in birds maintained in old quarters. 2. Penicillin failed to stimulate growth or decrease the *Clostridia* count in birds maintained in clean quarters where the *Clostridia* population was low. 3. The feeding of fecal *Clostridia* decreased the growth rate of birds reared in clean quarters. This decrease was overcome by addition of penicillin to the diet. The feeding of fecal *Clostridia* to birds reared in old quarters where the *Clostridia* population was high failed to depress growth. 5. Penicillin produced a growth stimulation only in cases where there was a decrease in the *Clostridia* count per gram of feces. This indicated that one explanation for antibiotics stimulating growth is through an action on the anaerobic (*Clostridia*) microflora.

---

1. Elam, J. F., Jacobs, R. L., Tidwell, W. L., Gee, L. L., and Couch, J. R., *J. Nutrition,* 1953, v49, 307.

2. Jacobs, R. L., Elam, J. F., Anderson, G. W., Gee, L. L., Fowler, Jean and Couch, J. R., *ibid.,* 1953, v51, 507.

3. Kiser, J. S., De Mello, G. C., Reichard, D. H., and Williams, J. H., *J. Infect. Dis.,* 1952, v90, 76.

4. Sieburth, J. M., Gutierrez, J., McGinnis, J., Stern, J. R., and Schneider, B. H., PROC. SOC. EXP. BIOL. AND MED., 1951, v76, 15.

5. Elam, J. F., Jacobs, R. L., Fowler, Jean, Hale, Fred, and Couch, J. R., *J. Animal Sci.,* 1953, v12, 819.

6. Coates, M. E., Dickinson, C. D., Harrison, G. F., Kon, S. K., Cummins, S. H., and Cuthbertson,

W. F. J., *Nature,* 1951, v168, 332.

7. Coates, M. E., Dickinson, C. D., Harrison, G. F., Kon, S. K., Porter, J. W. G., Cummins, S. H., and Cuthbertson, W. F. J., *J. Sci. Food Agric.,* 1952, v1, 43.

8. Bird, H. R., Lillie, R. J., Machlin, L. J., and Denton, C. A., *Proc. 9th World's Poultry Congress,* 1951, v2, 46.

9. Lillie, R. J., Sizemore, J. R., and Bird, H. R., *Poultry Sci.,* 1953, v32, 466.

10. Hill, D. C., Branion, H. D., Slinger, S. J., and Anderson, G. W., *ibid.,* 1953, v32, 462.

11. Elam, J. F., Gee, L. L., and Couch, J. R., Proc. Soc. Exp. Biol. and Med., 1951b, v78, 832.

12. ———, *ibid.,* 1951a, v77, 209.

Received March 31, 1954. P.S.E.B.M., 1954, v85.

Role of Magnesium Ions in Donath-Landsteiner Hemolytic Reaction of Paroxysmal Cold Hemoglobinuria.* (20981)

J. Albert Baxter[†] and William S. Jordan, Jr.[‡]

From the Departments of Preventive Medicine and of Medicine, School of Medicine, Western Reserve University, and University Hospitals, Cleveland, O.

The effect of certain metallic ions on complement activity has attracted considerable attention in recent years(1-5). The activity of guinea pig complement against sensitized sheep erythrocytes has been shown to depend on the concentration of Mg^{++} used in the reagents(1,6,7), and it has been stated(5) that calcium as well as magnesium is essential for this immune hemolysis. Other investigators, however, have found that these ions enhance but are not essential for the activity of rabbit(8) or human complements(9). While studying the mechanism of hemolysis in paroxysmal nocturnal hemoglobinuria, Harris, Jordan, Pillemer and Desforges(10) found that Mg^{++} is essential for the activity of the serum factor, or factors, which hemolyse the abnormal erythrocytes. No immune mechanism, however, has been demonstrated in this unusual human hemolytic system(11,12). Accordingly, the role of magnesium was studied in another human hemolytic system, that of paroxysmal cold hemoglobinuria (PCH), in which hemolysin and complement are involved.

The PCH system is unique among the hemolytic systems in that it requires the presence of complement both for antibody fixation in the cold phase of the Donath-Landsteiner reaction as well as for hemolysis in the warm phase(13). The experiments described in this paper examine the part that Mg^{++} plays in these two phases. The sheep cell hemolytic system was used to demonstrate any change which might have occurred in complement activity when Mg^{++} was removed from guinea pig serum by various methods.

Materials and methods. PCH sera. Sera from three patients with paroxysmal cold hemoglobinuria were used. Case No. 1 and case No. 2 have been described previously (13). Case No. 3 was seen in February, 1952, at Crile Veterans Administration Hospital through the courtesy of Dr. Norman Shumway. This 30-year-old colored male had had episodes of cold hemoglobinuria since 1945. Despite a number of courses of penicillin, he was still having frequent episodes of hemoglobinuria. Kahn and Kolmer tests were positive. The Donath-Landsteiner hemolysin titer was 1:4; the PCH antibody titer measured by the indirect Coombs test was 1:16. *Complement titration.* Lyophilized guinea pig serum was used, and was titered with a modification of the method described by Kabat and Mayer(14), using the Coleman Jr. spectrophotometer. The 50% unit of complement was determined by plotting the hemolysis curve on logarithmic paper. All titrations were made in duplicate, and repeated titration showed that the results were reproducible within 5%. *Buffer.* Barbital buffer(1) containing 1.5 x 10^{-3} M $CaCl_2$, but no Mg^{++}, was used in those experiments designed to test the effect of Mg^{++} depletion. To restore Mg^{++}, similar buffer containing 5 x 10^{-4} M Mg Cl_2 was used. *Removal of Mg^{++} from reagents.* Mg^{++} was removed from the serum specimens by dialysis, by resin treatment, or by a combination of both procedures. *I. Dialysis.* The serum was placed in cellulose tubing and dialysed against phosphate-buffered saline (pH 7.2) for 24 hours at 4°C, 100 ml of buffer being used for each 1 ml of serum dialysed.

---

* This investigation was supported in part by grants from the Brush Foundation, and Mr. Philip R. Mather.

[†] Study done during tenure of Crile Fellowship.

[‡] Markle Scholar in Medical Science.

II. Ion-exchange treatment. The sodium cycle of the cation exchange resin, Amberlite IRC-50, was used in all the ion-exchange experiments. Two methods of treatment were used. *A. Ion-exchange column.* A resin column was formed by drawing a narrow neck in a piece of 15 mm glass tubing. A small piece of glass wool was then placed in this neck and a suspension of the resin was poured on the wool. The tube was then placed in an upright position, so that the resin would form an even bed over the glass wool. One half ml of 50% resin in distilled water was used for each 1 ml of serum to be treated, and the column was washed three times with buffered saline before use. The exchange process was carried out at 4°C without suction. *B. Dialysis in presence of ion-exchange resin.* The serum was prepared as previously described for dialysis, but 0.5 ml of the 50% resin suspension was added to each 100 ml of buffered saline and dialysis carried out in the presence of resin for 24 hours at 4°C. The resin was kept in suspension by using a magnetic stirrer throughout the treatment procedure. *Quantitative magnesium determination.* Mg^{++} was determined by a modification of the titan yellow photocolorimetric method described by Orange and Rhein(15). By this method concentrations of between 0.065 μM and 1.0 μM per ml can be determined. *The Donath-Landsteiner test.* A 2.5% suspension of human "O" erythrocytes was standardized to give an optical density of 0.54 when 0.5 ml of the cell suspension was lysed with 2.5 ml of distilled water. This suspension was prepared daily from "O" cells stored in Alsever's solution for not longer than 3 days. For the test the following reagents were added to a tube in an ice bath: 1) 0.25 ml PCH serum; 2) 0.25 ml guinea pig serum (diluted 1:2); 3) 0.50 ml 2.5% "O" cell suspension. Following the addition of the reagents the tubes were shaken, chilled in an ice bath for 30 minutes, and then immediately placed in a 37°C water bath for 30 minutes. Following this incubation, 2.0 ml of barbital buffer was added to each tube, and after centrifugation, the supernates were read for hemolysis in 10 x 75 mm cuvettes at 550 mμ in the Coleman, Jr. spectrophotometer. *Indirect Coombs test.* This test was used in an attempt to quantitate the amount of antibody absorbed by the red cells. The method used was a slight modification of the method previously described [indirect (a)] (13).

Results. Effect of Mg^{++} removal on C' titer. Guinea pig serum was treated in 3 ways to determine the method which removed the most Mg^{++} with the least destruction of complement lytic activity. Table I shows the results of such a series of treatments using three different complement pools. The barbital buffer with Mg^{++} was found to contain 0.65 μM of Mg^{++} per ml, and that buffer without Mg^{++}, no measurable amount of Mg^{++}. Thus it is apparent that with the complement diluted 1:100 or 1:50, the buffer and not the complement would become the principal factor in controlling the amount of Mg^{++} present in the titration. For this reason it was felt safe to assume that in the titrations in the presence of Mg^{++} the concentration was about 0.65 μM per ml, and in the titration in the absence of Mg^{++} there was none or at least less than 0.065 μM per ml. Therefore, there was at least 10 times as much Mg^{++} present in the titrations with Mg^{++} as there was in those lacking this ion. An increase in the Mg^{++} concentration above 0.65 μM per ml failed to enhance the complement titer.

Of the various methods used to remove Mg^{++} from serum, passage through a resin column was found to be the most destructive to complement activity even after Ca^{++} and Mg^{++} had been restored to optimum levels. Dialysis in the presence of a resin was found to be less destructive of complement activity,

TABLE I. Effect of Mg^{++} Removal on Complement Titer of Guinea Pig Serum.

Serum pool	Serum treatment	μM of Mg^{++} /ml undiluted serum	Mg^{++} in reagents .65 μM/ml*	<0.065 μM/ml	% reduction due to Mg^{++} removal
			$C'H_{50}$ units/ml		
1	Untreated	1.01	455	327	28
	Resin exchanged	.13	323	323	0
	Dialysed	.47	394	289	27
	Dialysed with resin	.14	361	314	13
2	Untreated	1.06	565	413	27
	Resin exchanged	.12	378	269	29
	Dialysed	.55	459	335	27
	Dialysed with resin	.12	429	300	30
3	Untreated	.61	526	436	17
	Dialysed with resin	.14	343	258	25

* Mg^{++} in the reagents restored Mg^{++} concentration in all specimens to essentially same level. Thus differences in titer between treated and untreated specimens reflect effect of treatment procedure.

but as efficient as the resin column in removing Mg^{++}. Dialysis against buffered isotonic saline was the least destructive of complement activity, but also removed less Mg^{++} than the other methods used. In subsequent experiments, therefore, Mg^{++} was removed from serum by dialysis in the presence of resin.

Effect of Mg^{++} on PCH hemolysis. When at least 90% of the Mg^{++} was removed from the reagents used in the Donath-Landsteiner test, the amount of hemolysis was reduced by amounts varying between 45 to 86% (Table II). When de-ionized human serum was used as a complement source for the serum from case 2 there again was but a slight decrease in the amount of hemolysis. In the PCH hemolytic system, this decrease could have been caused by a reduction in the activity of the lytic components of complement or by a failure of the antibody to unite properly with the red cells.

The indirect Coombs test was used in order to measure the quantity of antibody fixed to the erythrocytes. When reagents lacking Mg^{++} were used, the indirect Coombs titer decreased 4-fold in 2 cases and 2-fold in one case, indicating a reduction in the amount of PCH antibody fixed to the red cells. Since this reduction might have been caused by damage to the antibody during treatment, the Donath-Landsteiner test was set up using PCH serum which had been dialysed with resin, all other reagents containing their original amounts of Mg^{++}. When this was done, it was found that the amount of hemolysis returned to within 10% of the value found prior to treatment of the serum. Thus, the reduction in the amount of PCH antibody fixed as well the decrease in the degree of hemolysis may be attributed to the effect of lack of Mg^{++} on complement activity.

It has been demonstrated that PCH hemolysis depends upon the presence of complement in both the cold and warm phases of the Donath-Landsteiner reaction(13). Accordingly, the need for Mg^{++} during the two phases was investigated, by washing erythrocytes once with a large volume of cold buffer after chilling in complement and sera from case No. 2 or case No. 3 and then adding new lots of complement prior to incubation at 37°C. When Mg^{++} was present in both phases, 93 to 97% hemolysis was obtained. The amount of hemolysis was reduced slightly to 84 to 87% when Mg^{++} was lacking in either the cold phase or the warm phase. As noted previously, hemolysis occurred even though Mg^{++} was absent in both phases.

Discussion. In the experiments reported above the amount of Mg^{++} in the reagents used for complement titration was reduced to very low levels while the amount of calcium was kept constant. Reduction in the amount of Mg^{++} did not prevent hemolysis from occurring in the sheep cell system. Although Mg^{++} was removed to such an extent that it could no longer be detected, the complement titer of guinea pig serum was reduced at most by about 30%. In a hemolytic sys-

TABLE II. Effect of Mg^{++} on PCH Hemolysis.

Case No.	Mg^{++} added		Mg^{++} removed		% reduction due to Mg^{++} removal
	μM Mg^{++}/ml*	% hemolysis	μM Mg^{++}/ml*	% hemolysis	
1 (Serum 1:2)	1.01	86	.052	12	86
2	.84	70	.044	35	50
3	.77	88	.075	48	45

* These values were calculated from quantitative magnesium determinations made on the reagents and thus represent the actual amount of magnesium in the test.

tem free of both Ca^{++} and Mg^{++}, Pillemer, Blum, Pensky and Lepow(9) found that the complement titer of human serum was decreased about 50%. The present study is in agreement with these results in indicating that while Mg^{++} enhances complement activity, it is not an absolute requirement for immune hemolysis.

In the PCH system Mg^{++} removal was not as complete, for in this system both human serum (PCH antibody) and guinea pig serum (complement) had to be used in low dilutions (13). The amount of hemolysis, however, was reduced to a greater extent than in the sheep cell system. This is not a surprising result as it is known that a large amount of complement is required for the lysis of human erythrocytes sensitized with PCH antibody (13). Further, it was shown that suboptimal amounts of Mg^{++} decreased slightly the amount of PCH antibody fixed on the red cells in the cold phase of the Donath-Landsteiner reaction. The addition of reagents containing Mg^{++} after the cold phase reduced the effect of a lack of Mg^{++} in the cold phase, but did not eliminate it. When Mg^{++} was present in the cold phase and reagents lacking Mg^{++} were used in the warm phase, the amount of hemolysis was less than obtained when Mg^{++} was present throughout. Thus Mg^{++} does not appear to be essential for hemolysis in the PCH hemolytic system, although reduction in the concentration of Mg^{++} decreases the activity of complement in both the cold and warm phases of the Donath-Landsteiner reaction.

Summary. 1. Mg^{++} was removed from guinea pig and human sera by dialysing the serum in the presence of an ion-exchange resin. 2. Complete or almost complete removal of Mg^{++} from reagents used in the sheep cell hemolytic system, with the concentration of Ca^{++} being maintained, diminished the complement titer of guinea pig serum by about one-fourth. 3. Antibody fixation was only slightly reduced when Mg^{++} was lacking in the cold phase of the Donath-Landsteiner reaction. Maximum hemolysis was obtained when Mg^{++} was present in both the cold and warm phases. 4. Mg^{++} does not appear to be essential for hemolysis in the Donath-Landsteiner reaction of paroxysmal cold hemoglobinuria.

---

1. Mayer, M. M., Osler, A. G., Bier, O. G., and Heidelberger, M., *J. Exp. Med.*, 1946, v84, 535.

2. Lepow, I. H., Pillemer, L., and Ratnoff, O. D , *ibid.*, 1953, v97, 573.

3. Levine, L., Cowan, K. M., Osler, A. G., and Mayer, M., *J. Immunol.*, 1953, v71, 359.

4. ———, *ibid.*, 1953, v71, 367.

5. Levine, L., Osler, A. G., and Mayer, M., *ibid.*, 1953, v71, 374.

6. Cernovodeanu, P., and Henri, V., *Compt. rend. Soc. de biol.*, 1906, v60, 571.

7. Kellogg, W. H., and Wells, L. A., *J. Lab. Clin. Med.*, 1926, v12, 153.

8. Maurer, P. H., and Weigle, W., *J. Immunol.*, 1953, v71, 284.

9. Pillemer, L., Blum, L., Pensky, J., and Lepow, I. H., *ibid.*, 1953, v71, 331.

10. Harris, J. W., Jordan, W. S., Jr., Pillemer, L., and Desforges, J. F., *J. Clin. Invest.*, 1951, v30, 646.

11. Ham, T. H., and Dingle, J. H., *ibid.*, 1939, v18, 657.

12. Crosby, W. H., *Blood*, 1953, v8, 444.

13. Jordan, W. S., Jr., Pillemer, L., and Dingle, J. H., *J. Clin. Invest.*, 1951, v30, 11.

14. Kabat, E. A., and Mayer, M. M., *Experimental Immunochemistry*, Springfield, Ill., C. C. Thomas., 1948.

15. Orange, M., and Rhein, H. C., *J. Biol. Chem.*, 1951, v189, 379.

Received March 12, 1954. P.S.E.B.M., 1954, v85.

Sperm Agglutinins in Human Semen and Blood. (20982)

Leo Wilson. (Introduced by Alfred J. Weil.)

From the Endocrine Gynecology Clinic, Bronx Hospital and the Department of Obstetrics and Gynecology, Morrisania City Hospital, New York City.

Antibodies against spermatozoa have been known to impair sperm functions(1-3). Such antibodies, however, have not previously been found under natural conditions and, particularly, not in man.* This is a report of 2 men whose spermatozoa agglutinate in the ejaculate. Antibodies were found in their seminal plasma and blood serum. These cases were encountered by the writer in the past year and a half during the routine clinical investigation of approximately 100 sterile couples.

Clinical observations. Case 1. White male, age 30, American Jewish descent. Family and personal medical history negative. A brother has one child. Blood group *AB*, $Rh_0(D)$ positive; no history of blood transfusions or injections. Married 10 years. Wife unable to conceive although in good health, her sterility investigation negative and no contraception used. Her blood group is *B*, $Rh_0(D)$ positive. She promptly became pregnant following artificial insemination of a donor's semen and is now in her last trimester. *Case 2.* White male, age 36, American Jewish descent. Family and personal medical history negative. A brother has 2 children and a sister has one child. Blood group *B*, $Rh_0(D)$ negative, *anti*-$Rh_0(D)$ negative. No history of blood transfusions or injections. Married 3 years. Wife unable to conceive although in good health, her sterility investigation negative and no contraception used. Her blood group is *O*, $Rh_0(D)$ positive. In both cases the semen was examined many times and consistently showed normal values for the ejaculate volume, sperm count, viscosity, initial motility, morphology and non-spermatozoal cellular content. However, these specimens always showed marked clumping of the spermatozoa and early loss of motility. It was therefore decided to study this phenomenon in detail.

Methods. All semen specimens were obtained by masturbation after a period of sexual abstinence of not less than 48 hours. The ejaculates were collected directly into clean, dry glass containers and, on several occasions, in 2 separate fractions (split-ejaculate). Sperm agglutination was detected by microscopic examination of a wet film prepared by laying a cover-slip over a drop of semen on a clean glass slide. The presence of agglutinins in the blood serum and seminal plasma was determined by mixing a drop of the respective fluid with a drop of fresh normal semen and examining the wet film microscopically for sperm clumping. As soon as the initial microscopic observation of sperm motility and agglutination was completed, the edges of the cover-slip were sealed with melted vaseline. The slides were re-examined at intervals up to 24 hours or until all spermatozoa were immobilized. The rapidity, degree and type of agglutination (head, tail or mixed), the influence of temperature (4°, 20°, and 37°C) and the relation between agglutination and sperm motility were noted. The latter was studied by a) observing the fresh ejaculate microscopically during liquefaction (while the spermatozoa were becoming motile), b) observing spermatozoa during post-coital and Kurzrok-Miller cervical mucus invasion tests, and c) comparing the behavior of normal motile and heat-immobilized spermatozoa (56°C for 30 minutes) in the presence of

---

*Kibrick *et al.* (*Fertil. and Steril.* 1952, v3, 435) reported agglutination of human spermatozoa by some normal sera of rabbits, guinea pigs and man in low dilutions. They used a macroscopic tube agglutination technic with gelatin. No significance was attributed to this observation which was considered non-specific. It was rare in dilutions over 1:4 in contrast to the titers up to 1:256000 obtained with potent anti-human-sperm rabbit's sera. Because of the different technics employed, it is not possible to compare the findings of these workers with the observations reported here. Spermatozoa were not agglutinated by normal human sera with the method used in this study.

blood and seminal agglutinins. Serial dilutions of the blood sera were made with isotonic saline. Complement was inactivated by heating serum and seminal plasma in a 56°C waterbath for one hour.

Absorption tests were performed as follows: equal amounts of dilute serum were placed in 2 small test tubes; fresh normal semen of high sperm count and active motility was added to one tube and an equal volume of isotonic saline to the other; after heating in a 37°C waterbath for one hour, the tubes were centrifuged and the supernates were tested for sperm agglutinins as described above.

Results. Agglutination of the spermatozoa of normal subjects was produced by the seminal plasma and blood serum of the 2 men who are the subject of this report. The microscopic appearance of the sperm clumps was identical with that seen in their ejaculates. Agglutination was not obtained with the seminal plasma or serum of 8 fertile men or the serum of 8 pregnant women.

The speed of agglutination varied from one minute to one hour depending upon the agglutinin titer, temperature and quality of sperm motility. The clumps continued to grow as long as sperm motility was preserved. However, even after 24 hours, a few motile spermatozoa remained unclumped.

As observed under the microscope, agglutination began in one of 3 ways: 1. *"Head" agglutination*: motile spermatozoa formed into groups of 2, 3 or 4, joined side by side with their heads in apposition and their tails separate. The tails soon became entangled with the tails or heads of other groups, thereby forming larger and larger clumps. Individual motile and immotile spermatozoa, as well as cellular and other debris, similarly became enmeshed in the clumps and contributed to their growth. 2. *"Tail" agglutination*: the tails of several motile spermatozoa became intertwined while their heads remained separate at the periphery forming a rosette-like cluster. Other spermatozoa swam into or were caught in the network of tails and thus the size of the clump increased. 3. *"Mixed" agglutination*: the tail of one spermatozoon became attached to the head of another. Soon other spermatozoa were entangled and a clump formed.

FIG. 1. Sperm agglutination in ejaculate of Case 1. Unstained wet film preparation (50 ×).

Regardless of the original type of agglutination, within a short time all the larger clumps had a similar appearance. This was predominantly that of "tail" agglutination with free sperm heads visible at the periphery. Occasionally a particularly active spermatozoon was seen detaching itself from the edge of a clump by its own effort. The agglutinated spermatozoa evidently were not firmly bound to each other (at least during the first hour) since the clumps were partially disrupted by removing the cover-slip from the slide. The clumps were completely dispersed by bubbling air through the ejaculate but reagglutination took place in a few minutes. The new clumps, however, did not attain their previous size probably because some of the spermatozoa, in the meantime, had lost their motility. The appearance of sperm agglutination at 50 × and 215 × magnification is shown in Fig. 1 and 3. For comparison, nonagglutinated spermatozoa are shown in Fig. 2 and 4.

Sperm motility. Agglutination occurred only in the presence of motile spermatozoa and none was seen in the fresh ejaculate until liquefaction began and active sperm movement developed (5 to 15 minutes). Within a short time, however, the agglutinated spermatozoa became sluggish and finally lost their motility. This was true of the majority of the spermatozoa and, particularly, those deep inside the clumps. Nevertheless, a small number on the periphery and some of those remaining free continued to exhibit fairly active movement for as long as 24 hours if the preparation was sealed with vaseline to prevent drying and was kept at a temperature between 4° and 20°C.

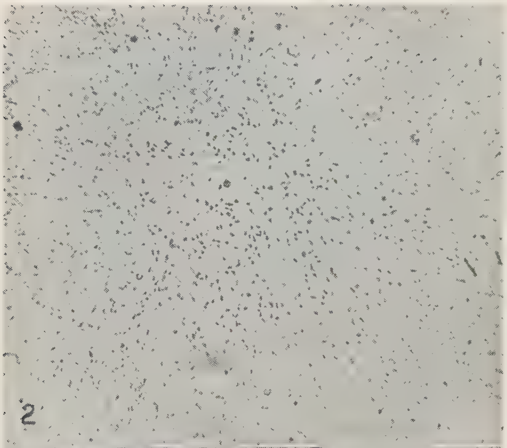
Temperature. Agglutination occurred at room temperature (20°C) but was more rapid at 37°C due to the increased sperm motility at the higher temperature. Low temperatures retarded clumping by reducing sperm motility while temperatures appreciably above 37°C immobilized the spermatozoa and halted agglutination.

Post-coital and cervical mucus invasion tests. Post-coital examination of the cervical mucus, performed during the pre-ovulatory phase of the menstrual cycle and 3 to 48 hours after intercourse or artificial insemination, demonstrated the presence of unusually few spermatozoa and the motility of these was sluggish or absent. Agglutinated spermatozoa were not seen in the cervical mucus probably because penetration was prevented by agglutination and impaired motility. Cervical mucus invasion tests gave similar results: spermatozoa passed through the interface but quickly lost their motility. Sperm clumps were present in the seminal portion but not in the mucus.

The split-ejaculate. The first portion of the ejaculate contained many more spermatozoa than the second portion and agglutination was seen in both.

Immunologic data. Clumping took place in dilutions of the blood serum up to 1:80 in *Case 1* and 1:20 in *Case 2*. Agglutination was not affected by inactivating the complement in the serum and seminal plasma. Sera kept at 4°C for 3 months did not lose their capacity to clump spermatozoa. The agglutinins in the blood serum were removed by absorption. Agglutination did not occur when normal spermatozoa and normal seminal plasma (or blood serum) of subjects with different ABO and $Rh_0(D)$ blood groups were mixed.

Discussion. No satisfactory explanation for the presence of sperm agglutinins in the blood and semen in these 2 cases can be given at present. Clumping has been observed in infected ejaculates(4) and has been produced



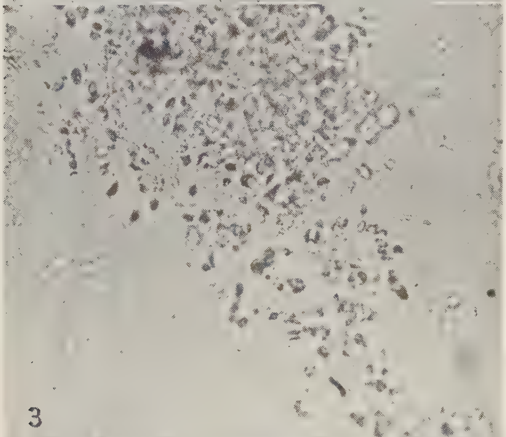


FIG. 2. Normal semen showing absence of sperm agglutination (50 ×).

FIG. 3. Higher magnification of Fig. 1 (215 ×). The dominant appearance is of "tail" agglutination with sperm heads free at periphery of clump.

FIG. 4. Higher magnification of Fig. 2 (215 ×).

experimentally in human or animal semen by acids, alkalis, carbon dioxide, salts of heavy metals, dyes, extracts of eggs, bacteria and antisera developed by injecting spermatozoa of the same or different species(5). None of these agents appears to be involved in the cases reported here.

Summary. The presence of sperm agglutinins in the seminal plasma and blood serum of 2 sterile men whose spermatozoa agglutinate in the ejaculate is reported. The sera have antibody titers of 1:80 and 1:20, respectively. Head, tail, and mixed varieties of agglutination are seen in the early stages, but tail agglutination predominates in the larger clumps. Agglutination occurs only in the presence of motile spermatozoa and none is seen in the fresh ejaculate until liquefaction begins and active sperm movement develops. Temperature influences agglutination by its effect on sperm motility. The motility of the clumped spermatozoa is impaired with consequent loss of the ability to penetrate cervical mucus. The clumps can be completely dispersed by mechanical agitation after which the spermatozoa reagglutinate although not quite to the original extent. Complement inactivation does not influence agglutination. The antibody can be removed by absorption.

The author is indebted to Dr. Alfred J. Weil, Director of Bacteriology of the Bronx Hospital, for advice and encouragement and to Dr. Magda Rona Dacso, Pathologist of the Morrisania City Hospital, for providing laboratory facilities for this study.

1. Landsteiner, K., *Centralbl. f. Bakt.*, 1899, v25, 546.

2. Metchnikoff, E., *Ann. Inst. Pasteur,* 1900, v14, 1.

3. Freund, J., Lipton, M. M., and Thompson, G. E., *J. Exp. Med.*, 1953, v97, 711.

4. Lane-Roberts, C., *et al., Sterility and Impaired Fertility,* 2nd ed. 1948, Paul B. Hoeber, N. Y., and London, p. 74.

5. Rosenthal, L., Proc. Soc. Exp. Biol. and Med., 1931, v28, 827.

Received March 24, 1954. P.S.E.B.M., 1954, v85.

Combined Effects of X-Irradiation and Testosterone Propionate on Accessory Sex Organs. (20983)

W. Fleischmann* and M. Nimaroff.

From the Veterans Administration Hospital, Oteen, N. C.

A temporary inhibition of cell division appears to be a general action of radiation, having been demonstrated in a great variety of cells. If the cells are prevented from dividing, but continue to grow at the normal rate, then the size of the cells will increase instead of their number. When division is delayed by irradiation it is of interest to determine at which stage or stages the retardation occurs. The answer to these questions can most readily be obtained in the case of materials in which the cells develop synchronously, so that all the cells can be irradiated at the same stage. It is obvious, that this experimental condition cannot as a rule be fulfilled in growing tissues as they are composed of cells at different stages of cell division, so that it is not possible to choose a single stage to be irradiated(1). Experiments in which a single stage was irradiated were performed by Henshaw(2), who treated unfertilized eggs of the sea urchin, *Arbacia punctulata,* with x-rays and studied the delay of first cleavage following subsequent fertilization. In these experiments all cells were in the resting stage during irradiation and cell division was induced subsequently by fertilization.

It occurred to us that irradiation of the secondary sex organs (seminal vesicles and prostate) of the castrate male rat and subsequent stimulation by an androgen offers a unique opportunity for an analogous study on the effect of radiation on mammalian cells.

---

* Present address: Veterans Administration Hospital, Crump Boulevard, Memphis, Tenn.

TABLE I. Effect of Combined Irradiation and Treatment with TP. All rats received colchicine 7½ hours before death.

Group	No. of rats	TP, hr	X-ray, hr	Accessories, wt in mg Mean	S.D.	Access. hypertrophy	Access. mitoses	Pelvic skin mitoses	Head skin mitoses
A	7	—	—	24.8	7.0	0	0	0	+
B	12	48	—	80.7	27.2	+	++	+	+
C	9	48	48½	65.4	13.8	+	0	0	+
D	7	48	168	72.0	6.5	+	++	+	+

Statistical comparison of weights

Groups compared	P
A-B	<.01
A-C	"
A-D	"
B-C	.1
C-D	.3

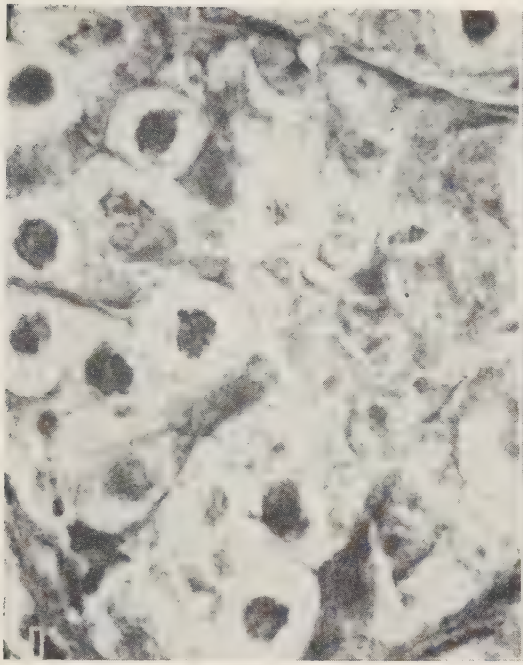
The accessory sex organs of the castrate male rat do not grow unless stimulated by androgens. Treatment of the castrate male rat with androgens is followed by hyperplasia and mitotic activity of the cells of the accessories. The abundance of mitotic figures in the stimulated accessories is in striking contrast to their absence in the resting organs. This effect on mitosis can be emphasized by the use of colchicine(3-5). A preliminary study showed that the weight response of the accessories of the castrate male rat to testosterone propionate (TP) is not inhibited by irradiation of the accessories with x-ray. Even if doses of 10000 r are used the accessories respond with an increase in weight to subsequent treatment with TP(6).

Materials and methods. Male rats of the Wistar strain were castrated at the age of about 30 days and were used not earlier than one month after castration. X-ray was given under amytal sodium anesthesia. Irradiation was given through a portal centered over the pelvis. The diameter of this portal was 3½ cm. The rats received 3000 r (120 KV, 8 ma, filter 1 mm A1; 98 r/min.). Except for the pelvic region the rats were shielded by 2 mm lead. TP (Perandren Ciba) was given dissolved in peanut oil in doses of 3 mg subcutaneously. Colchicine, 3 mg/kg body weight, was given in aqueous solution 7½ hours before termination of the experiment. The animals were sacrificed with amytal sodium. The accessories were dissected out, weighed to the nearest mg and fixed in 10% formalin. Skin from the irradiated suprapubic region and from the shielded head was also fixed. Sections were stained either with dilute Harris hematoxylin or with Nicolle's carbol thionin stain.

Results. The results of the experiments are listed in Table I. From this table the following conclusions can be drawn. Irradiation in the dose given has no or little effect on the growth response of the accessories due to TP under the conditions of the experiment. However, if TP is given immediately after irradiation mitotic activity is suppressed. The cells in group C show hypertrophy without mitosis. The difference in appearance between the non-irradiated and the irradiated tissues is quite striking; the epithelial cells of the non-irradiated accessories show numerous mitoses whereas mitotic figures are completely absent in the irradiated tissues. The contrast can be easily seen by comparing Fig. 1 and 2.

This difference is obvious only in the accessories of rats killed 48 hours after irradiation, followed by TP (Group C). If the rats are killed 24 hours after administration of TP no mitotic figures can be seen. Both the irradiated and the non-irradiated specimens show hypertrophy only. This was to be expected as the onset of mitosis in the prostate of the castrate rat had been determined at about 36 hours after administration of TP (5). On the other hand if the rats are killed 72 hours after administration of TP mitoses are numerous both in the irradiated and the non-irradiated specimens.

If TP is not administered immediately



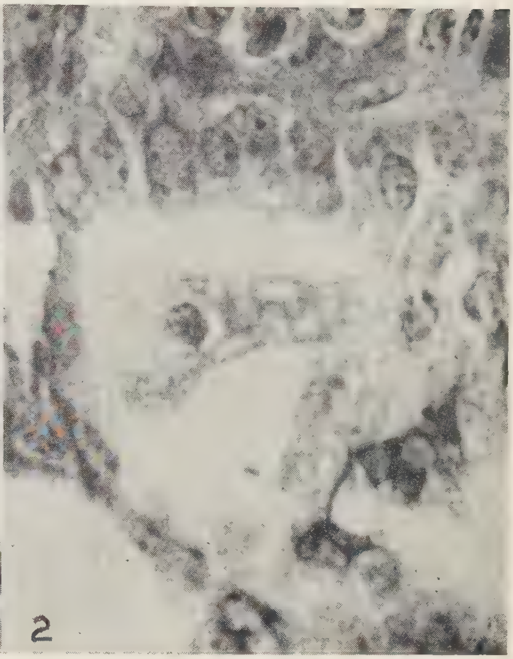


FIG. 1 (left). Seminal vesicle. Rat treated with TP 48 hr before death, with colchicine 7½ hr before death. (Group B.) Fixed in 10% formalin. Stained with carbol thionin. Note numerous typical colchicine mitoses. × 550.

FIG. 2 (right). Seminal vesicle. Same treatment as in Fig. 1, except that pelvic region received x-ray immediately preceding treatment with TP. (Group C.) Note absence of mitotic figures. × 550.

but 120 hours (5 days) after irradiation (Group D) mitotic activity appears to be normal as judged from the presence of numerous mitotic figures.

Sections were taken from the irradiated part of the skin to make sure that the pelvic region had received adequate irradiation. Table I shows that in all cases in which mitotic activity was absent in the accessories it was absent also in the pelvic skin. Sections from the skin of the shielded head served as controls. All of these sections showed numerous mitoses.

Discussion. The experiments indicate that the onset of mitosis can be delayed by irradiation of resting tissues. They show furthermore that this inhibiting effect is only transient. Recovery from the inhibiting effect of x-rays on mitosis can take place in the resting tissue as evidenced by the finding of mitoses in Group D. This finding is comparable to results of experiments on the eggs of the sea urchin mentioned above(2). Exposure of eggs of *Arbacia punctulata* to large doses of irradiation leads to delay of the first cleavage after subsequent fertilization. If an interval of time is allowed between the end of treatment and the moment of insemination this delayed effect is lost or reduced as a function of time. In our experiments the mitosis-inhibiting effect due to irradiation is lost or at least reduced if an interval of 5 days is allowed between time of irradiation and of stimulation with TP. It is of interest, that the delaying effect of x-irradiation on cell division is lost as a function of time in resting cells. This holds true both for unfertilized ova of invertebrates and for accessory sex organs of castrate vertebrates.

Jadassohn *et al.*(7) studied the combined effect of estrogens and x-irradiation on a target organ. One nipple of the male guinea pig was irradiated and both nipples subsequently treated topically with a solution of an estrogen preparation. Mitotic activity was estimated using the colchicine technic. The action of the estrogen administered after irradiation produced a simple hypertrophy involving both nuclei and cytoplasm associated with inhibition of mitosis. These results are essentially in agreement with those described in this paper.

Our experiments are reported here in a preliminary fashion. As indicated in an earlier note(6) they are part of a project to investigate the influence of x-rays on the formation of chromosomal material. The accessory sex organs of the castrate rat seem to be a suitable object for studies of this kind as their growth response to TP is associated with rapid formation of desoxyribonucleic acid as indicated by tracer studies using P^{32}(8).

Summary. X-irradiation of the accessory sex organs of the castrate male rat and immediate subsequent treatment with testosterone propionate results in cellular hypertrophy with delayed onset of mitosis under the conditions of the experiment outlined above. If an interval of 5 days is allowed between irradiation and administration of testosterone propionate this delaying effect is lost.

We would like to thank Messrs. Edgar S. Greene, John G. Cohoe and Harley H. Nanney for their technical assistance.

1. Lea, D. E., *Actions of Radiations on Living Cells,* Cambridge University Press, 1949.
2. Henshaw, P. S., *Am. J. Roentgenol.,* 1940, v43, 913.
3. Fleischmann, W., and Kann, S., *Biochem. Z.* 1938, v296, 373.
4. Tislowitz, R., *Endocrinology,* 1939, v25, 749.
5. Burkhart, E. Z., PROC. SOC. EXP. BIOL. AND MED., 1939, v40, 137.
6. Fleischmann, W., and Brackin, J. T., Jr., *Endocrinology,* 1954, v54, 227.
7. Jadassohn, W., Bujard, E., Pillard, R., Wenger, P., and Gaudin, R., *Acta Radiol.,* 1950, v34, 469.
8. Fleischmann, W., and Fleischmann, S. Kann, *J. Mt. Sinai Hosp., N. Y.,* 1952, v19, 228.

Received March 26, 1954. P.S.E.B.M., 1954, v85.

Ion Exchange and Recrystallization in Fixation of Ca^{45} in the Rabbit's Skeleton.*† (20984)

J. S. ARNOLD AND W. S. S. JEE. (Introduced by Thomas F. Dougherty.)

From the Radiobiology Laboratory and Department of Anatomy, College of Medicine University of Utah, Salt Lake City.

Ion exchange has been demonstrated to be the principal mechanism for the fixation of radiocalcium and phosphate in bone, both *in vitro*(2,3) and *in vivo*(4-6). Calcium ions, in a solution of an ionized calcium salt, exchange with other calcium ions on the surface of bone crystals(3). Thus, under suitable conditions, it is possible to replace the calcium ions on the crystal surfaces with those of a calcium solution. In the studies herein reported Ca^{45} which remained on the crystal surfaces at various intervals after administration was determined by *in vitro* ion exchange removal in non-radioactive calcium solutions.

The purpose of these studies was to examine directly the role of ion exchange, crystallization and recrystallization in the fixation of calcium in newly formed and mature bone. No attempt will be made to review the concepts of surface chemistry of bone at this time. Recently an excellent review of the field has been made by Neuman(7). If all of a given amount of Ca^{45} were fixed in bone by ion exchange and remained on the crystal surfaces, then nearly all of the Ca^{45} should be removable by ion exchange with non-radioactive calcium solutions. On the other hand, if all of the Ca^{45} fixed in bone was incorporated into newly forming crystals, not more than 50% of the Ca^{45} should be removable by surface ion exchange.‡ The actual process of skeletal calcium fixation in a growing or mature ani-

---

[*] Preliminary results were presented at the Sixty-fourth Annual Meeting of Am. Physiol. Soc.(1).

[†] Supported in part by AEC Contract and the U. S. Public Health Service.

mal is a complicated combination of ion exchange and crystallization. In addition, there is the continuous process of recrystallization taking place in both newly formed and old bone(5,8,9). Formation of new crystals and recrystallization should reduce the *in vitro* exchangeability of *in vivo* fixed Ca^{45} in bone. These studies were in part designed to evaluate the rate and relative importance of these processes in calcium metabolism of bone. (Newman considers recrystallization as "a slow equilibrium of the crystal interior with the solution phase"(7).) After Ca^{45} is fixed on the surface of a formed bone crystal, recrystallization is the process which prevents its removal by *in vitro* ion exchange. These studies are, in part, comparable to those of Neuman using radiophosphate in rats(9). However, rabbits were used rather than rats because of their larger skeleton and Haversian system bone structure characteristic of large mammals. Further, Ca^{45} was used rather than P^{32} labeled phosphate because of its longer radioactive half-life and its greater specificity in relation to the inorganic phase of bone metabolism. Overall exchangeability of Ca^{45} was determined by applying beta-ray measurement technics to the bone sections before and after removal of the exchangeable Ca^{45}.

Method. Four 6 lb female, New Zealand white rabbits were given 0.5 mc/kg of Ca^{45} as the chloride (a total of 0.5 mg of calcium) intravenously. Animals were sacrificed at 1 hour, 24 hours, 1 week and 3 weeks following a single intravenous administration of Ca^{45}. The third and fourth lumbar vertebral bodies were longitudinally halved. One half of each vertebral body was fixed in acetone for 48 hours. The other halves were fixed in 4% formaldehyde solution containing ~1% sodium borate. Formalin fixed tissues were washed for 12 hours in 2 changes of distilled water prior to dehydration in alcohols. Dehydration of acetone-fixed tissue was completed in one change of acetone, prior to placing in 50:50 ether-alcohol. Five to 10 μ sections of the undecalcified bone embedded in plasticized nitrocellulose were prepared by a technic that we have previously reported (10). Sections were mounted on gelatinized glass slides and the embedding medium was then removed in absolute acetone. Sections were treated in .01 M CaAc[§] containing .01 M boric acid and ~.0006 M Na ions. Control sections were treated with the buffer solution alone as well as distilled water. The results reported are on acetone-fixed bone sections except when formalin-fixed material is specified. Sections were treated with buffer solution prior to CaAc treatment as well as after CaAc treatment. All treatments were carried out under the following constant conditions: (I) Temperature, 18°C; (II) time, 1 hr; (III) solution volume, 50 ml; (IV) agitation, constant and slow; and (V) pH 7.7-7.8 (except for the distilled water whose pH was 6.2-6.5). Serial sections were so distributed that all comparative studies on a single animal were as representative as possible of the bone of the vertebral bodies. A group of 4 representative bone sections from an animal were treated simultaneously. Four groups of sections, one from each animal of the four sacrifice periods were treated at the same time and under the same conditions. Each study was repeated 3 or more times. Other groups of bone sections from the animals of the four sacrifice intervals were treated in buffered CaAc for periods varying from 2 minutes to 96 hours. Individual sections were counted in a windowless proportional counter before and after each treatment. The loss of activity[‖] from individual sections was calculated and corrected for radioactive decay. All counting data was normalized to a Ca^{45} standard counted before and after each section. In order to evaluate the possibility of small microscopic fragments of bone being lost from the bone sections in the process of exposure to agitated solutions, 6 different

---

[‡] *In vitro* studies indicate that the fraction of phosphate in a bone crystal which is present on the surface is less than 15% in old mature bone and 50% or greater in newly deposited bone(8). All evidence to date indicates that this relation would also exist for calcium ions qualitatively and probably quantitatively.

[§] In the following text, for reasons of brevity, all solutions referred to as CaAc are .01 M calcium acetate containing the above stated borate buffer.

[‖] Activity is the number of beta disintegrations detected per unit of time under the conditions of measurement.

TABLE I. Ca^{45} Desorption from Bone Sections by 1 Hr Treatments in CaAc and Buffer Solutions.

Sacrifice time following Ca^{45} admin.	% of Ca^{45} desorbed by treatment in:	
	CaAc	Buffer
1 hr	56.20 ± .94	27.47 ±1.06
24 "	29.73 ±1.05	11.60 ± .96
7 days	16.60 ± .57	5.15 ± .74
21 "	15.10 ± .78	7.00 ±1.23

treatment solutions containing the large quantities of Ca^{45} leached from bone sections were filtered through fine fritted glass discs. In no case was any detectable activity present on the surface of the discs. These results would indicate that no appreciable loss of Ca^{45} occurred due to fragmentation of the bone under the experimental conditions. When fragmentation did occur it was always visably obvious and the results correspondingly unreasonable.

Results. When sections of bone from the different animals were treated in CaAc solution, the fractional loss of Ca^{45} content decreased with increasing sacrifice period. This loss was 56.2 ± .94%[¶] from the bone of the rabbit sacrificed at 1 hour. Table I and the solid line of Fig. 1 show the decrease in the fraction of Ca^{45} leached[**] in CaAc as a function of time following Ca^{45} administration.[††] Control treatment of bone sections in the buffer solution alone produced a loss of 27.47 ± 1.06% of the initial Ca^{45} from the bone of the 1 hr animal. The loss of Ca^{45} in buffer solution from the bones of the animals of the 4 sacrifice intervals are also listed in Table I. No statistically significant difference was observed in the data between Ca^{45} loss in buffer and distilled water. When groups of sections were treated first in buffer solution and then in CaAc, the total loss was found to be comparable to treatment in CaAc alone. The bone of the 1 hour animal regularly lost 25 (s[‡‡] = 2)% and 33 (s = 3)% of the initial activity in the first and second treatments, respectively. When formalin-fixed bone sections from the same animal (which had been washed in distilled water during processing) were treated first in buffer solution followed by CaAc, the loss of activity was only 16 (s = 2)% in the buffer solution; however, the loss in CaAc solution was statistically the same as that which occurred in acetone-fixed sections.

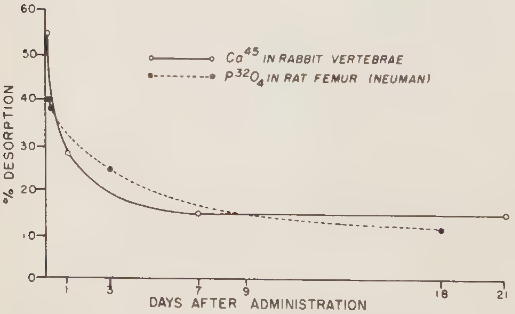


FIG. 1. Plot of % of Ca^{45} desorbed from bone section by 1 hr treatment in CaAc as a function of the time following administration, and comparison to radiophosphate data of Neuman(8).

These studies indicate that Ca^{45} is present in 3 or more forms in the acetone-fixed bone sections of the animals sacrificed at 1 and 24 hours after Ca^{45} administration. These forms are as follows: 1. That which is lost in distilled water or buffer solution, and 2. That which is exchangeable with calcium ions over and above the fraction lost in water or buffer, and 3. That which is not removable by *in vitro* ion exchange. The data indicates that the first 2 fractions are independent of one another, as demonstrated by the fact that removal of the first does not appreciably affect removal of the second. For convenience, the Ca^{45} fraction lost in distilled water or buffer will be referred to here as the loosely bound fraction, and that fraction which is removed only by ion exchange is referred to as the firmly bound fraction. From all studies, the loosely bound fraction constitutes 25 (s = 2)% and the firmly bound fraction 32 (s = 3)% of the initial Ca^{45} in the bone sections of the 1 hour animals. The loosely bound fractions represent a little less than ½ and the firmly bound fraction a little more than ½ of the Ca^{45} lost from the bone section by 1 hour CaAc treatment. This same relation exists for the relative magnitudes of these fractions in the bone of the 1 and 24 hour animals. The data are not sufficiently good for the 1 and 3 week animals to make a definite statement because of the increased error in measuring small differences. The results obtained on formalin-fixed bone sections parallel those obtained on acetone-fixed material. The exception to this being that the loosely bound fraction was slightly smaller in the formalin-fixed than acetone-fixed bone. This decrease in the loosely bound fraction of formalin-fixed bone may be due to its partial loss in the course of distilled water washing during pre-embedding processing. Rough calculations indicate that 10-20% of the Ca^{45} in the bone sections of the animal sacrificed after 1 hour should be present in the bone marrow, blood, etc. It is very likely that soft tissue Ca^{45} makes up a large component of the loosely bound Ca^{45} fraction in the 1 hour animal.

[¶] Plus or minus values given are standard errors of loss from individual samples in groups of bone sections.

[**] Leaching is a general term denoting loss by any mechanism.

[††] When Ca^{45} is exchanged onto bone section *in vitro* in the course of 1 hour in radioactive $CaCl_2$ solution 77 ± 4% of it is removed in a similar treatment in non-radioactive $CaCl_2$(11). This would indicate that the treatment of CaAc under the conditions used removed approximately 3/4 of the Ca^{45} present in crystal surfaces. From this study it is apparent that the results reported in Table I as loss in CaAc treatment are slightly smaller than the true value of the total exchangeable Ca^{45} fraction.

[‡‡] s = the standard deviation from the mean value of all evaluating experiments.

When bone sections were treated in CaAc for increasing periods of time, there was an initial rapid followed by a slow progressive loss of the Ca^{45} from the bone sections of the 4 sacrifice intervals (Fig. II).

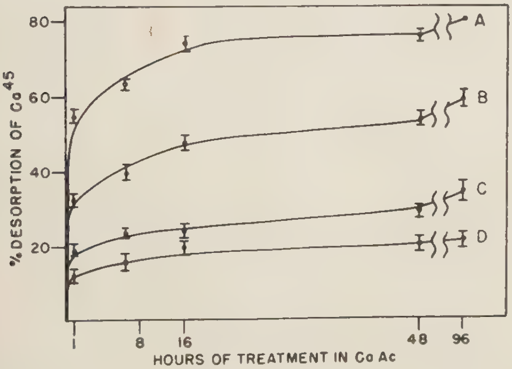


FIG. 2. Desorption of Ca^{45} from bone sections, as a function of time of treatment in CaAc. Sacrifice times of animals following Ca^{45} administration are: A. 1 hr, B. 24 hr, C. 1 wk, and D. 3 wk.

The shape of the curves of the rate of Ca^{45} loss may be explained by the following: (a) Ion exchange of crystal surface Ca^{45} with solution phase calcium is an exponential function with respect to time(3) and (b) *in vitro* recrystallization is probably occurring continually(8), uncovering previously buried Ca^{45}. It is thought that this process of recrystallization does not produce an appreciable renewal of crystal surfaces in most systems during the first 72 hours of exposure to a solution phase(9). There is unquestionably some overlap in the loss due to pure exchange of crystal surface Ca^{45} and that due to release of buried Ca^{45} by recrystallization. The time interval of 1 hour was arbitrarily chosen for purposes of experimental convenience, as well as obtaining results well situated on the plateau of loss. The difference in the fractional loss of Ca^{45} from bone sections of animals representing the four sacrifice intervals become increasingly apparent with increasing time of treatment in CaAc. Differences exist between the loss from the bone of the 1 and 3 week sacrificed animals after the 96 hour treatment, which was not as apparent after the 1 hour period. The interpretation of the 96 hour values for the four animals is difficult in view of the occurrence of *in vitro* recrystallization. These data indicate that *in vivo* fixed Ca^{45} progressively becomes more deeply incorporated in the lattices of bone crystals as time passes.

Discussion. Neuman demonstrated that radioactive phosphorus which was fixed *in vivo* in the skeletons of young and old rats was rapidly made unavailable to *in vitro* removal by ion exchange technic(9). The work herein reported demonstrates that Ca^{45} deposited *in vivo* in the slowly growing skeleton of rabbits is likewise made unavailable to removal by ion exchange. The similarity of results is demonstrated in Fig. 1 where both sets of data are shown.

From the reported data it is concluded that approximately 50% of the Ca^{45} present in the bone of the rabbit sacrificed 1 hour following intravenous Ca^{45} had been incorporated in a non-exchangeable form of bone mineral. At 24 hours and 21 days following Ca^{45} administration this non-exchangeable fraction had increased to 70 and 85%, respectively. From these data, it may be concluded that a large fraction of the crystal surface calcium ions are constantly being converted to nonexchangeable intracrystalline positions in bone as a whole.

In separate investigations paralleling the reported experiments, the exchangeability of *in vivo* fixed Ca^{45} has been determined in separately studied areas of mature and forming bone by quantitative radioautographic technics(11,12). These studies show that deposited Ca^{45} becomes unavailable to *in vitro* ion exchange in mature bone at almost the same rate as that of newly formed bone. Thus the reported data for the change in availability of deposited Ca^{45} to ion exchange on bone as a whole are equally applicable to both areas of mature and forming bone (separately).

It is unmistakably apparent that the vast majority of the Ca^{45} ions in bone are initially fixed *in vivo* by the process of ion exchange (7). The *in vitro* exchangeable ions are usually considered to be those contained in the first layer or surfaces of bone crystals(7). The processes by which *in vivo* fixed Ca^{45} becomes unavailable to *in vitro* ion exchange removal (placed in intracrystalline positions) are (I) incorporation in newly forming crystals and (II) entrapment by the poorly understood process of recrystallization. The 2 processes are unquestionably occurring simultaneously in areas of forming bone, and probably only the latter is taking place in areas of mature bone(9). One may consider the rate at which the *in vivo* fixed Ca^{45} becomes unavailable to *in vitro* ion exchange removal as the rate of transformation of crystal surface ions to intracrystalline positions. When this is done with the reported data and that of Neuman(9), it is apparent that the half time of the crystal surface to intracrystalline transformation rate must be less than one hour. This is a surprisingly rapid rate for such a transformation to be taking place in the solid inorganic phase of bone where metabolic activity has frequently been considered to be relatively inactive.

The problem of dissolution versus exchange loss of Ca^{45} from bone sections treated in various manners must be considered. It was not possible in these studies to quantitatively measure the loss of calcium from treated bone sections.[§§] Since approximately 20% of the Ca^{45} fixed on bone sections by *in vitro* exchange is invariably lost by treatment in distilled water(11), it is assumed that 20% of the crystal surface bound calcium is quite soluble. This extremely soluble fraction may represent calcium which is present in the recently described hydration layer of bone crystals(13). It is not unreasonable to assume that the loss of Ca^{45} during treatment of bone sections in buffer solution, in the reported studies, was substantially due to simple dissolving of calcium from crystal surfaces. This type of loss should be considerably smaller in CaAc treatment since the solubility of hydroxypatite is dependent upon the calcium and phosphate ion produced (among other things(7)). This problem will be considered more in detail in separately reported studies (11).

Summary. Undecalcified bone sections of rabbits sacrificed at intervals between one hour and 3 weeks following Ca^{45} administration were treated in calcium acetate solutions. This procedure was designed to determine what portion of Ca^{45} remains on crystal surface of bone as a function of time following administration. The data are interpreted as indicating that recrystallization and crystallization processes are removing calcium to non-exchangeable positions at extremely rapid rates. At one hour following Ca^{45} administration, ½ of the isotope fixed in bone is no longer available to removal by ion exchange technic.

The authors are indebted to the director of the Radiobiology Laboratory, Dr. John Z. Bowers, whose cooperation and guiding interest facilitated this work. The technical assistance of Patricia Hancey and the statistical consultation and work of Danuta Hiz and Mrs. Chamberlain are particularly appreciated. One

---

[§§] Analyses done on bone section and treatment solutions reveal that less than 10% (.01 mg) of the total calcium of the bone was lost in buffer treatment.

of the authors indebted to Dr. C. P. Leblond of McGill University for his stimulating influence in this field of endeavor.

---

1. Arnold, J. S., *Fed. Proc.*, 1952, v7, 5.
2. Falkenheim, M., Neuman, W. F., and Hodge, H. C., *J. Biol. Chem.*, 1947, v169, 713.
3. Falkenheim, M., Underwood, E. D., and Hodge, H. C., *ibid.*, 1951, v188, 805.
4. Hevesy, G. V., Levi, H. B., and Rebbe, O. H., *Biochem. J.*, 1940, v34, 532.
5. Neuman, W. F., and Riley, R., *J. Biol. Chem.*, 1947, v168, 545.
6. Singer, L., and Armstrong, W. D., Proc. Soc. Exp. Biol. and Med., 1951, v76, 229.
7. Neuman, W. F., and Neuman, M. W., *Chem. Rev.*, 1953, v53, 1.
8. Neuman, W. F., and Mulryan, B. J., *J. Biol. Chem.*, 1950, v185, 705.
9. ———, *ibid.*, 1952, v195, 843.
10. Arnold, J. S., and Jee, W. S. S., in press.
11. Arnold, J. S., in preparation.
12. Arnold, J. S., Johnson, K. E., and Jee, W. S. S., in press.
13. Neuman, W. F., Toribara, T. Y., and Mulryan, B. J., Atomic Energy Report, 1952, UR-230.

Received March 29, 1954. P.S.E.B.M., 1954, v85.

Effect of Propylene Glycol on Methemoglobin-Forming Capacity of p-Chloroacetanilide in Rat and Guinea Pig.* (20985)

Vera C. Glocklin. (Introduced by F. E. Ray.)

From the Cancer Research Laboratory, University of Florida, Gainesville, Fla.

Propylene glycol has been used extensively as a non-toxic and metabolically inert solvent for the oral or parenteral administration of various water-insoluble compounds to animals and man. Studies concerning gastric secretion in rats(1), however, have shown rapid secretion by the stomach of Toluidine Blue when injected in aqueous solution, but no secretion when injected in propylene glycol solution. The supposedly innocuous nature of propylene glycol has also been disputed: a marked hypertensive effect in cats, rats, and rabbits(2), and LD_{50} doses in rats of 13 ml/kilo intraperitoneally and 28 ml/kilo orally(3) have been reported. In view of these findings, the effect of p-chloroacetanilide on the blood of rats and guinea pigs in presence and absence of propylene glycol was investigated.

Methods. Adult male Wistar rats, 200-400 g, and adult female guinea pigs, 500-630 g, were used. All animals received tap water and food *ad libitum.* The rats were maintained on Purina Laboratory Chow pellets and the guinea pigs on Purina Rabbit Laboratory Chow pellets. The test doses (Table I) were administered by intraperitoneal injection. p-Chloroacetanilide, only slightly soluble in water, was suspended in a finely divided state in 0.1% methylcellulose solution; the methylcellulose being used for its wetting property. Two hours after injection, 2 ml blood samples were obtained by heart puncture under light ether anesthesia. Heparinized syringes were used to prevent blood coagulation (3 mg sodium heparin/ml distilled water). Cell volume to plasma volume ratios were determined by the hematocrit method of Wintrobe and Lansberg(4); total blood pigment by the method of Sahli(5); and methemoglobin by the method of Michel and Harris(6).

Results. The results are summarized in Table I. A dual effect on the blood is evident. The injection of propylene glycol produced an increase in hematocrit over the normal value. There was a corresponding increase in total blood pigment which indicated loss of fluid from the blood. This increase in hematocrit was uniform in both species and was not influenced by the presence of p-chloroacetanilide. On the other hand, p-chloroacetanilide produced methemoglobinemia in both species in presence and absence of propylene glycol. The methemoglobin-producing effect of a propylene glycol solution of p-chloroacetanilide, was greater in the rat (8.09%) than in

---

* Supported by contract with the U. S. Atomic Energy Commission, Division of Biology and Medicine.

the guinea pig (4.21%); and when administered in aqueous suspension, the methemoglobinemia greatly increased and the species difference was accentuated (rat, 40.8%; guinea pig, 6.89%).

All rats receiving propylene glycol alone became markedly apathetic and showed ataxia, particularly of the hind legs. Only one of 4 guinea pigs showed slight ataxia. The ataxia is possibly caused by lactic acid accumulation in the muscles, more evident in the active rat than in the more docile guinea pig. The increased viscosity of the blood could reduce its oxygen-carrying capacity enough to cause oxygen debt in the muscles. Furthermore, since propylene glycol has been shown to exhibit a glycogenic effect(9), it may competitively inhibit the reconversion of muscle lactic acid to glycogen. Propylene glycol also has been shown to be converted to lactic acid *in vitro*(10). All animals receiving p-chloroacetanilide in propylene glycol were ataxic. Most of the rats receiving the p-chloroacetanilide suspension became cyanotic and prostrate. The guinea pigs receiving the suspension were ataxic but not cyanotic, which is understandable since the methemoglobin did not reach the level (18-20%) necessary for observable cyanosis(11).

The reason for the modification of the methemoglobin-producing effect of p-chloroacetanilide by propylene glycol is not clear. The propylene glycol may reduce the rate of absorption and thus the availability of the p-chloroacetanilide at any one time; yet, distribution studies on the Cl^{36} labeled compound have shown ready absorption(12). Conversely, the propylene glycol may act as a biochemical poison on the enzyme mechanisms necessary to produce the methemoglobin-forming metabolite of the p-chloroacetanilide. It is evident in any case, however, that propylene glycol should be avoided whenever possible as a solvent for biological work, and that results attributed to compounds administered in propylene glycol solution should be carefully interpreted since the propylene glycol may alter the physiological response.

Summary. Rats and guinea pigs injected with propylene glycol, 4 ml/kg, with or without p-chloroacetanilide, showed a uniform in-

TABLE I. Effect of 100 mg/Kilo p-Chloroacetanilide and 4 ml/Kilo Propylene Glycol on Blood of Rat and Guinea Pig.

Group and treatment*	No. animals		Hematocrit,† %		Total pigment, g %		Methemoglobin, % of total pigment	
	Rat	Guinea pig	Rat	Guinea pig	Rat	Guinea pig	Rat	Guinea pig
1. 100 mg/kg pCAA in 4 ml/kg PG	9	4	55.7 ± 3.66	50.4 ± 2.64	19.8 ± 1.82	18.5 ± .42	8.09 ± 3.76	4.21 ± .06
2. 4 ml/kg PG	7	4	51.0 ± 13.45	51.8 ± 5.68	20.2 ± .36	18.4 ± 2.41	1.51 ± .36	2.21 ± .04
3. 100 mg/kg pCAA in 4 ml/kg MC	10	4	45.4 ± 2.47	43.8 ± 2.63	16.9 ± .41	16.7 ± 1.49	40.8 ± 15.78	6.89 ± .09
4. 4 ml/kg MC	3		46.3 ± 5.30		17.4 ± .40		1.75 ± .07	

* pCAA = p-Chloroacetanilide; PG = propylene glycol; MC = 0.1% aqueous methylcellulose solution.

† Comparison of totals of groups 1 and 2 with 3 within each species was statistically significant $p < 0.02$. Normal hematocrits are 45% for the guinea pig(7) and 42% for the rat(8).

crease in hematocrit to 51% as compared with a normal value of 42-45%. Animals receiving no propylene glycol showed no increase in hematocrit above the normal value. p-Chloroacetanilide, 100 mg/kg, dissolved in propylene glycol, 4 ml/kg, produced methemoglobinemia in both species; to a greater extent in the rat than in the guinea pig: 8.1% and 4.2% of the total blood pigment, respectively. Propylene glycol alone, 4 ml/kg, produced no increase in methemoglobin above normal range: 1.5% for rats, and 2.2% for guinea pigs. p-Chloroacetanilide, 100 mg/kg, in aqueous suspension increased the methemoglobinemia in both species; the increase more pronounced in the rat than in the guinea pig: 40.8% and 6.9% of the total blood pigment, respectively. These results indicate a decrease in production of the methemoglobin-producing metabolite of p-chloroacetanilide in the presence of propylene glycol. It is suggested that results attributed to a compound administered in propylene glycol solution should be carefully interpreted since the propylene glycol may make a marked contribution to the physiological activity of the mixture.

1. Klein, M., Argus, M. F., and Ray, F. E., *Brit. J. Cancer*, 1953, v7, 264.

2. Croxatto, H., Vasquez, W., Badia, W., and Jarpa, S., *Bol. soc. biol.*, Santiago, Chile, 1950, v8, 10.

3. Thomas, J. F., Kesel, R., and Hodge, H. C., *J. Ind. Hyg. Toxicol.*, 1949, v31, 256.

4. Gradwohl, R. B. H., *Clinical Laboratory Methods and Diagnosis*, St. Louis (Mosby), 4th ed., 1948, v1, p529.

5. ———, *ibid.*, 1948, v1, p520.

6. Michel, H. O., and Harris, J. S., *J. Lab. Clin. Med.*, 1940, v25, 445.

7. Gardner, M. V., *J. Franklin Inst.*, 1947, v243, 77.

8. ———, *ibid.*, 1947, v243, 498.

9. Newman, H. W., van Winkle, W., Kennedy, N. K., and Morton, M. C., *J. Pharm. Exp. Therap.*, 1940, v68, 194.

10. Rudney, H., *Arch. Biochem.*, 1950, v29, 231.

11. Cantarow, A, and Trumper, M, *Clinical Biochemistry*, Philadelphia (Saunders), 3rd ed. 1945, p98.

12. Gryder, J. M., Argus, M. F., and Ray, F. E., *Fed. Proc.*, 1954, v13, 221.

Received March 8, 1954. P.S.E.B.M., 1954, v85.

Synovial Fluid. I. Comparison of Sodium and Potassium Concentrations in Normal and Diseased Joint Fluid.* (20986)

K. LEMONE YIELDING, DAVID PLATT,[†] AND HOWARD L. HOLLEY.
(Introduced by Ward Pigman.)

From the Departments of Medicine and Biochemistry, Medical College of Alabama, Birmingham, Ala.

The finding of excess synovial fluid in various disease states has prompted us to investigate the sodium and potassium concentrations of fluids obtained from arthritic joints and compare them with those obtained from the normal. Previous investigations on synovial fluid have utilized material obtained from cattle(1,2), horses(3), dogs(4), edema effusion(5), and human autopsy material (2,6,7).

Material and methods. The synovial fluid samples were aspirated from the knee joints of 10 normal living subjects, 10 autopsy cases, 25 patients with effusions in untreated acute rheumatoid arthritis, and 5 individuals with a diagnosis of osteoarthritis with effusion. After repeated failure to obtain fluid from living subjects, it was found that by allowing the subjects to remain inactive for 8-12 hours prior to the procedure, volumes of fluid up to one cc could be obtained from the knee joint with only moderate difficulty. A 20-gauge needle was introduced under the medial border of the patella directly into the joint cavity. Prior infiltration of the skin with 1% procaine made the procedure relatively painless. The

---

* Supported by grants from the Am. Med. Assn., Chicago, Ill., and the National Institutes of Arthritis and Metabolic Diseases of the National Institutes of Health, Public Health Service, Bethesda, Md.

[†] Research Fellow of the Arthritis and Rheumatism Foundation.

TABLE I. Na and K Concentrations in mEq/L in Normal and Diseased Joints.

Cases	No.	Na	K
Normal living	10	136.1±1.63	4.0±.25
" autopsy	10	133.7±3.36	4.8±.13
Rheumatoid arthritis	25	143.6± .98*	4.6±.09
Osteoarthritis	5	136.9±1.95	4.1±.10

Synovial fluid/serum ratios for Na and K

	Na	K
Normal living	.93±.011	1.08±.09
Rheumatoid arthritis	.99±.010*	1.03±.04
Osteoarthritis	.94±.014	.95±.12

* Significantly different from normal.

fluid obtained from individuals with rheumatoid arthritis was first allowed to clot and then centrifuged before analysis. Sodium and potassium concentrations were determined using the Beckman DU flame photometer. In order to achieve the necessary uniform viscosities and aspiration rates for all samples, Wydase‡ was added to each solution at the rate of 50 TR units per cc of synovial fluid.

Results. Comparative results are shown in Table I. The average sodium concentration in normal synovial fluid was 136.1 mEq/l, with a fluid/serum ratio of 0.93 (0.89-0.98). The ratios for potassium ranged from 0.70-1.70, with an average concentration in the joint fluid of 4.0 mEq/l. Average sodium and potassium values from autopsy fluids were 133.7 and 4.9, respectively.

Synovial fluids obtained from the patients with rheumatoid arthritis revealed average sodium and potassium values of 143.6 and 4.6 mEq/l, with fluid/serum ratios of 0.99 (0.95-1.03) and 1.03 (0.92-1.19). Fluid obtained from osteoarthritic joints had average concentrations of 136.9 for sodium and 4.1 for potassium and fluid serum ratios of 0.94 sodium and 0.95 potassium, respectively. Statistical analysis of the data revealed a difference of 3.95 standard errors between the mean sodium concentration in normal synovial fluid and that of rheumatoid arthritics. In view of the wide range in fluid/serum ratio for potassium in normal fluids, little significance could be attributed to this phase of our investigation.

Discussion. Ropes and Bauer have determined the sodium and potassium distribution ratios between serum and synovial fluid in cattle(2). The fluid/serum ratio for sodium was 0.93 and that for potassium was 0.75. Their findings appear to correlate closely with the sodium values obtained in this study of normal and autopsy material.

The increase in the sodium concentration in the joint fluid of patients with rheumatoid arthritis as compared to that found in normal fluids appears to be statistically significant. This increase in synovial fluid sodium concentration can be considered to be of more significance since the serum values were essentially equal in normal subjects and those with rheumatoid arthritis. This is doubly substantiated by a previous study of serum sodium and potassium values in rheumatoid arthritis (8), in which they were essentially equal to the reported normals(9).

There appear to be at least 3 possible factors affecting the alteration in the electrolyte concentrations of synovial fluid in diseased states; *i.e.,* alteration of protein content, qualitative and quantitative alteration of the polysaccharide component of the fluid, and changes in permeability of the synovial membrane. It has been shown electrophoretically that the total protein content and especially that of the globulin fraction is increased in the joint fluid in rheumatoid arthritis(10). More recently it has also been shown that there are extensive qualitative alterations in the protein components as evidenced by the changes in their mobilities seen in the electrophoretic pattern(11).

Conclusions. 1. Data have been presented on the sodium and potassium concentrations of normal human synovial fluid. From the values obtained it is apparent that the normal values for sodium previously reported on material obtained from cattle joints compares favorably with those found in the living human subject and in postmortem fluids. The potassium values were quite variable, using the present technics. In the synovial fluid obtained from rheumatoid arthritics, there was an increase in sodium concentration with a corresponding increase in the synovial fluid/serum ratio for sodium. 2. Technics are described for the collection and analysis of synovial fluid.

---

‡ Trade Name—Hyaluronidase—Wyeth Laboratories, Philadelphia, Pa.

---

1. Bauer, W., Bennett, G. A., Marble, A., and Claflin, D., *J. Exp. Med.,* 1930, v52, 835.

2. Bauer, W., Ropes, M. W., and Waine, H., *Physiol. Rev.,* 1940, v20, 272.

3. Hare, T., and Cohen, H., *Proc. Roy. Soc. Med.,* 1929, v22, 1121.

4. Yielding, K. L., and Rosano, C. T., Proc. Soc. Exp. Biol. and Med., 1951, v78, 54.

5. Boots, R. H., and Cullen, G. E., *J. Exp. Med.,* 1922, v36, 405.

6. Fisher, A. G. T., *Chronic (Non-Tuberculous) Arthritis,* The MacMillan Co., N. Y., 1929, 241 pp.

7. Horiye, K., *Virchow's Arch. Path. Anat.,* 1924, v251, 649.

8. Yielding, K. L., and Hogan, R. S., unpublished data.

9. Elliott, H. C., Jr., and Holley, H. L., *Am. J. Clin. Path.,* 1951, v21, 831.

10. Perlmann, G. E., and Kaufman, D. F., *J. Clin. Invest.,* 1946, v25, 931.

11. Platt, D., unpublished data.

Received March 23, 1954. P.S.E.B.M., 1954, v85.

Effects of Storage on Eastern Equine Encephalitis Virus. (20987)

Roy W. Chamberlain and R. K. Sikes. (Introduced by Morris Schaeffer.)

From the Communicable Disease Center, Public Health Service, U. S. Department of Health, Education, and Welfare, Montgomery, Ala.

Because of a lack of adequate information on the preservation of virus in field and laboratory specimens, an investigation was undertaken to explore, in part, the effects of various storage conditions upon eastern equine encephalitis virus (EEE). Some of the studies were designed to determine effects of different insect killing methods and storage temperatures upon EEE virus in infected, intact mosquitoes. Others were intended to measure the effects of carbon dioxide gas in dry ice storage upon virus in intact mosquitoes and mouse brains as well as mosquito and mouse brain suspensions. Stabilizing properties of several types of diluents on virus-containing mosquito and mouse brain suspensions were also investigated.

Materials and methods. The mosquitoes (*Aedes aegypti*) were infected with a recently isolated strain of EEE by feeding upon infected chicks and used after 12 days extrinsic incubation at 80°F. Twenty-five specimens from the infected pool inoculated into mice were all found to contain virus, which indicated uniform infection of the pool. Pools of 5 mosquitoes were used for each unit of the intact mosquito studies. After storage, each pool of 5 specimens was ground in 1.0 ml of 0.1% bovine albumin-buffered saline diluent (pH 7.2) and centrifuged 10 minutes at 2500 RPM. The supernatant was decanted into vials in an ice bath, 2.0 mg of streptomycin and 1000 units of penicillin per ml were added and serial 10-fold dilutions made in cold 0.1% bovine albumin-saline. These dilutions were inoculated immediately via the intracerebral route into 3-week-old CFW mice to determine the virus titers, with the suspension of 5 mosquitoes in 1.0 ml considered as concentrated material or 10^0 dilution. Virus titers in all tables are expressed as the inverse log of the dilution killing half the animals inoculated. When mosquito suspensions rather than intact mosquitoes were stored, they likewise were prepared in the ratio of 5 mosquitoes per ml of diluent. Infected mouse brains were stored as 10% suspensions and as intact brain tissue.

Experimental. Effects of various insect killing methods upon EEE virus in infected mosquitoes. To determine whether the most common methods of killing field-caught mosquitoes affected EEE virus they might contain, infected mosquitoes were killed in the following ways: 1) Exposed to ether fumes for 2 minutes. 2) Exposed to chloroform fumes for 2 minutes. 3) Exposed to chloroform fumes for 2 minutes, plus wetting of one specimen of each pool of 5 with chloroform. 4) Quick-frozen on dry ice in contact with

TABLE I. Effects of Various Methods of Mosquito Killing upon Virus in EEE-Infected *Aedes aegypti* Specimens, 5 Lots.

Killing method	Titers, 5 mosquitoes/ml Sublots: a	b	c	d	e	f
Ether fumes	5.5	5.5	5.5	5.5	5.0	5.3
Chloroform fumes	5.3	5.3	4.8	5.3	5.3	5.3
Idem + wetting	4.7	4.3	5.3	3.7	4.3	broken
Quick-freezing + CO_2 gas	5.3	4.5	5.5	5.5	5.3	5.3
Quick-freezing	4.7	5.0	5.0	4.5	5.3	5.3

CO_2 gas for 10 minutes. 5) Quick-frozen at dry ice temperature but not in contact with CO_2 gas (controls).

To test each of the above killing methods for virus-inactivating effects, 6 sublots of 5 specimens each were flame-sealed in soft glass ampules and stored on dry ice 4 months before virus levels were measured. The results (Table I) reveal no evidence that virus inactivation occurred when mosquitoes were killed by methods 1), 2), 4), and 5). A suggestion of some deleterious effect was observed in method 3) where the actual wetting of one specimen of every sublot of 5 deliberately introduced free chloroform into each ampule.

Effects of storage temperature upon EEE virus in intact quick-frozen mosquitoes. Infected mosquitoes were killed by quick-freezing, then flame-sealed in ampules and stored for a 4-month period under the following different conditions: (1) –70°C without fluctuation. (2) –70°C with 13 one- to 3-minute thawings at weekly intervals. (3) –70°C with one 6-hour thawing at room temperature (25°C) after 1 month's storage. (4) –70°C with an 11-hour interval at room temperature (6 hours after 1 month's storage and 5 hours after 2 months' storage). (5) –70°C with a 17-hour interval at room temperature (6 hours after 1 month's storage, 5 hours after 2 months, and 6 hours after 3 months. (6) Fluctuation between –5°C or –15°C to –50°C weekly on the top rack of a poorly maintained dry ice chest. (7) –20°C in a mechanical freezer. (8) Fluctuation between 0 and –20°C 2-5 times weekly in the freezing compartment of a household refrigerator in use in the laboratory. Controls were not placed in storage, but were titered immediately after quick-freezing. All tests were run in quintuplicate.

After storage under these conditions, the mosquito lots were ground, diluted, and titered. The results are shown in Table II.

TABLE II. Effects of Various Temperature Conditions upon EEE Titers in Ampuled, Intact *Aedes aegypti* during 4 Months' Storage, 8 Lots.

Storage conditions	Titers, 5 mosquitoes/ml Sublots a	b	c	d	e
Unstored controls	4.8	4.5	4.0	4.0	4.8
–70°C, constant	5.0	5.7	4.7	5.0	5.3
" , with 13 brief thaws	4.3	3.7	broken	4.2	4.3
" , " 6-hr thaw	2.7	3.2	2.0	3.5	3.2
" , " 11-hr "	2.8	2.3	1.7	2.0	2.5
" , " 17-hr "	1.3	1.3	0.6	1.5	0.7
Fluctuations, –5 to –50°C	3.2	3.4	3.3	3.7	3.3
–20°C, constant	4.8	4.2	4.3	4.0	4.3
Fluctuations, 0 to –20°C	<.0[*]	.0[†]	1.5	.7	.0

[*] No mice died after inoculation of the most concentrated material (5 mosquitoes ground in 1.0 ml is considered the 10^{-0} dilution).

[†] Half the mice died when inoculated with the most concentrated material.

At a constant temperature of –70°C, no drop in virus titer occurred. A constant temperature of –20°C appeared to give nearly comparable results, with titers only about half a log lower than in the –70° series, and on a par with the titers of unstored control specimens. Storage on the top rack in a dry ice chest, with fluctuations below the freezing point, resulted in a loss of about 1 to 1½ logs in titer. Following storage in the freezing compartment of a household refrigerator in use in the laboratory a loss of nearly all the virus in the test mosquitoes occurred.

Infected mosquitoes withstood 13 brief thawings from –70°C to near room temperature at weekly intervals with little effect on the virus they contained. However, longer

TABLE III. Effect of CO_2 Gas upon EEE Virus in Intact *Aedes aegypti* Mosquitoes Stored 4 Months on Dry Ice, 3 Experimental Lots.

Type of seal	Titer, 5 mosquitoes/ml Sublots a	b	c	d	e
Unstored controls	5.3	4.2	4.2	4.7	5.0
Glass sealed	4.7	5.5	5.0	4.5	5.0
Rubber stoppered, taped	5.5	5.3	5.0	5.5	5.3
Open ampules	4.5	5.0	4.5	4.5	4.7

TABLE IV. Effect of CO_2 Gas and Type of Diluent upon EEE Virus in Suspensions of *Aedes aegypti* Mosquitoes Stored 4 Months on Dry Ice.

Type of seal	Titers after storage in various diluents			
	Distilled water	Saline	0.1% bovine albumin	30% horse serum
Unstored control	3.0, 3.3, <3.0	4.3, 4.3, 4.0	4.2, 5.3, 4.2	5.0, 4.7, 4.0
Flame-sealed ampules	1.5, 2.3, 1.0	2.5, 2.5, 2.7	3.0, 2.5, 3.2	5.2, 4.7, 5.0
Open ampules	1.2, 1.8, 1.7	3.3, 2.8, 3.0	3.2, 2.3, 3.3	5.3, 5.5, 5.3

periods of thawing had more drastic effects. After 6 hours at room temperature there was drop in titer of approximately 1.5-2.0 logs; after 11 hours, about 2.0-2.5; and after 17 hours, about 3.0-3.5.

Effect of CO_2 gas upon EEE virus in intact infected mosquitoes stored on dry ice. Infected mosquitoes were stored 4 months on dry ice either in flame-sealed glass ampules, open ampules, or in test tubes sealed with tightly fitting rubber stoppers bound with 3 rounds of 1-inch adhesive tape. Control mosquitoes were ground and titered immediately after quick-freezing, the others after the storage period. All tests were run in quintuplicate. The results (Table III) revealed no significant differences among the different lots of specimens, regardless of degree of exposure to carbon dioxide gas.

Effect of CO_2 gas and type of diluent upon EEE virus in suspensions of infected mosquitoes stored on dry ice. Infected mosquitoes were ground in distilled water, saline buffered at pH 7.2, 0.1% bovine albumin in buffered saline, and 30% normal horse serum in buffered saline. A portion of each suspension was titrated in triplicate immediately; the remainder of each was dispensed in triplicate into ampules which were then either flame-sealed or left unsealed, and stored 4 months on dry ice before titering.

The results (Table IV) failed to show any adverse effects of CO_2 gas upon the virus. The 30% horse serum appeared to be the superior diluent, while the 0.1% bovine albumin-buffered saline and buffered saline alone were about intermediate in stabilizing power, and distilled water alone very poor.

Effect of CO_2 gas upon EEE virus in intact mouse brain tissue stored on dry ice. Three-week-old CFW mice were inoculated intracerebrally with 1000 LD_{50} each of EEE virus. Six were sacrificed when prostrate and the brains removed and divided into right and left halves. Left halves of 3 and right halves of another 3 were flame-sealed individually in Wassermann tubes as controls. The remaining right and left halves were placed unsealed in similar tubes. All were stored on dry ice for 4 months, after which time a 10% suspension was prepared of each half brain in 0.1% bovine albumin-buffered saline and titrated intracerebrally in mice. The results, given in Table V, reveal no significant differences in titers of those portions of brain exposed to CO_2 gas during storage and those not exposed.

Effect of CO_2 gas and type of diluent upon EEE virus in 10% mouse brain suspensions stored on dry ice. Ten per cent mouse brain suspensions were prepared in distilled water, saline buffered at pH 7.2, 0.1% bovine albumin in buffered saline, and 30% normal horse serum in buffered saline, using a pool of 3 EEE-infected brains for each suspension. Part of the material from each suspension was titered in duplicate immediately as an unstored control. The remainder was dispensed in triplicate in 1.0 ml amounts in ampules and stored flame-sealed or unsealed on dry ice for 4 months before titration.

The results (Table VI) reveal little difference in titers of the different brain suspensions, regardless of type of diluent or exposure to CO_2 gas during the test period.

TABLE V. Effect of CO_2 Gas upon EEE Virus in Intact Mouse Brain Tissue (Brain Halves) Stored 4 Months on Dry Ice.

Brain half	EEE virus titer per brain half	
	CO_2 free (A)	CO_2 exposed (B)
Left (A), right (B)	8.2	7.5
Idem	7.7	7.3
"	8.0	8.0
Right (A), left (B)	7.7	7.5
Idem	7.8	7.7
"	8.0	7.8

TABLE VI. Effect of CO_2 Gas and Type of Diluent upon EEE Virus in 10% Mouse Brain Suspensions Stored 4 Months on Dry Ice.

	Titers after storage in various diluents			
Type of seal	Distilled water	Saline	0.1% bovine albumin	30% horse serum
Unstored control	7.8, 8.2	7.8, 8.8	8.7, 8.6	8.2, 8.7
Flame-sealed ampules	8.5, 8.2, 8.3	9.0, 8.7, 8.0	9.0, 8.5, 9.3	7.8, 9.3, 8.2
Open ampules	8.2, 7.7, 8.0	7.0, 8.0, 8.5	8.0, 8.5, 8.0	8.3, 8.5, 8.2

Summary. A 4-month study on the influence of certain factors upon eastern equine encephalitis virus in stored mosquitoes and brain tissue revealed the following: (1) Killing of EEE-infected mosquitoes by brief exposure to ether or chloroform fumes had no ill effects upon the virus they contained. (2) A constant temperature of –70°C or –20°C was found best for preserving EEE virus in intact, infected mosquitoes. Several hours at room temperature caused loss of viability of most of the virus present, as did fluctuations 2 to 5 times weekly from 0°C to –20°C. Fluctuations in temperature below the freezing point also caused some drop in virus titer. (3) Carbon dioxide gas in an open type dry ice chest had no apparent deleterious effect upon EEE virus in infected, intact mosquitoes, intact mouse brain tissue, or mosquito and mouse brain suspensions in distilled water, buffered saline, 0.1% bovine albumin, or 30% normal horse serum. (4) Distilled water was an unsuitable diluent for suspensions of EEE-infected mosquitoes. Buffered saline and 0.1% bovine albumin both gave better, but still rather poor results. Thirty per cent normal horse serum proved superior, since with it no drop in virus titer was observed. (5) All the above diluents gave satisfactory results in 10% EEE-infected mouse brain suspensions during the 4-month test period.

Received March 29, 1954. P.S.E.B.M., 1954, v85.

Use of a Cation Exchange Resin for Isolation of Influenza Virus. (20988)

Kenneth Takemoto.* (Introduced by Dorland J. Davis.)
From the National Microbiological Institute, National Institutes of Health, Public Health Service, U. S. Department of Health, Education and Welfare.

The adsorption on and elution from ion exchange resins of a number of different viruses has been demonstrated by several workers. The partial purification of several neurotropic viruses was achieved by Muller (1) by the use of a cationic resin. An anionic resin was used by Lo Grippo(2) for the purification of the Lansing strain of poliomyelitis, and the extraction of poliomyelitis virus from human fecal material. That influenza virus can be adsorbed and eluted from cation resins has been shown by Muller and Rose(3), and Puck and Sagik(4). The studies presented here were undertaken to determine whether unadapted influenza virus from human sources behaves in a similar manner, and whether ion exchange resins can be used for the isolation of influenza virus.

Materials and methods. A cation exchange resin, Nalcite HCR-X12, 50-100 mesh,[†] was converted from the acid to the sodium form by several washings of 10% NaCl until the pH of the supernate was the same as that of the salt solution before addition to the resin. It was then washed with distilled water until washings no longer produced a precipitate when $AgNO_3$ was added. Resin which had been used was washed 3 to 5 times with distilled water then kept in a 56°C water bath for several days in 10% NaCl. This served to elute and inactivate any virus adsorbed

---

[*] Public Health Service Post-doctoral Research Fellow of the National Microbiological Institute.

[†] National Aluminate Corp., Chicago, Ill.

to the resin.

A preliminary study was made to determine whether an early passage, egg-adapted strain of influenza A virus (A/Va./11/53) isolated during the winter epidemic of 1953 could be adsorbed on and eluted from the cation resin, and whether the recovered virus remained in an active form.

Methods. Five ml of a 1/5 dilution of this egg-passage virus were added to 80 g wet resin in 250-ml flasks, shaken and kept at 4°C for 2 hours with gentle shaking every 20 minutes. The supernates were then withdrawn and tested for infectivity by intra-allantoic inoculation of 11-day-old embryos and the allantoic fluids from the embryos tested for hemagglutinins after 48 hour incubation. Elution of the virus was carried out by adding 5 ml of 10% NaCl to the resin, and keeping the flasks at 4°C for 2 hours with shaking every 20 minutes. The eluates were removed at the end of this period and infectivity titers determined by inoculating 11-day-old chick embryos intra-allantoically with serial 10-fold dilutions of the eluates, 6 embryos per dilution. Fifty per cent end points were calculated by the Reed and Muench method(5). Infectivity titers were also determined on the original 1/5 suspension which was further diluted to compensate for water contained within the wet resin. The water content of wet resin was found to be approximately 0.5 ml/g. Throat washings and swabs were obtained in sterile skim milk or beef heart infusion broth during the epidemic period in January and February 1953, in connection with epidemiological studies(6). They were preserved at –50°C to –70°C for 8-10 months before testing with resin. The routine procedure for influenza virus isolation as carried on in this laboratory consisted of intra-amniotic inoculation of 0.2 ml of the sample in 11-day-old chick embryos, incubation for 5 days at 37°C, after which the lungs, allantoic and amniotic fluids were harvested. The lungs were ground in alundum and suspended in the allantoic and amniotic fluids, and a second passage carried out by inoculating 0.2 ml of the suspension intra-amniotically into 11-day-old chick embryos. After 48 hours incubation at 37°C, living eggs were chilled overnight at 4°C. Allantoic and amniotic fluids were then collected and tested for hemagglutination with 0.4% chicken and guinea pig cells. Virus isolations involving the use of resin were conducted by adding 5 ml of throat sample to 30-40 g of wet resin in flasks. After gentle shaking, the flasks were kept at 4°C for 2 hours and shaken every 15-20 minutes. The supernates were then withdrawn and 2.5 ml of 10% NaCl added to the flasks. After 2 hours elution at 4°C the eluates were removed and 2.5 mg of streptomycin and 500 units of penicillin were added per ml of eluate. Eleven-day-old embryos were inoculated intra-amniotically with the eluates, each embryo receiving 0.2 ml. The procedure thereafter was the same as that described above.

Results. Table I presents the data of an experiment on the determination of infective virus recovered after adsorption and elution from the cation resin of early passage, egg-adapted influenza virus. Five aliquots of the same diluted allantoic fluid were treated separately and titers determined simultaneously. Virus adsorption to the cation resin is usually complete and cannot be detected in the supernate either by hemagglutination or infectivity tests. The ID_{50} of the treated aliquots varied from $10^{-2.60}$ to $10^{-3.50}$ compared to $10^{-3.65}$ for the untreated, indicating a loss not exceeding 10-fold. Puck and Sagik(4) had previously shown that they could recover virus from cation resins with hemagglutination titers the same as that of the original suspension.

Table II shows the results of virus isolations attempted on 106 throat samples selected by 2 separate categories. The 33 samples in Group A were untested samples taken

TABLE I. Hemagglutination and Infectivity Titers of Influenza Virus Treated with Cationic Resin. 5 treated aliquots.

	Hemagglutination titers Before adsorption	After adsorption	50% egg infectivity dose Supernate	Eluate
	1/16	0	0	$10^{-2.74}$
	”	0	0	$10^{-3.43}$
	”	0	0	$10^{-2.60}$
	”	0	0	$10^{-3.50}$
	”	0	0	$10^{-3.25}$
Control	”			$10^{-3.65}$

TABLE II. Comparison of Influenza Virus Isolations by Routine and Cation Resin Methods.

		No. isolations				
Group	No. samples	Routine	Resin	Both methods	Routine + Resin −	Routine − Resin +
A	33	13	17	19	2	6
B	73	4	10	10	0	6

from individuals from whom virus had been isolated in another sample by the routine method. Thirteen of these proved to be positive by the routine method as compared to 17 by treatment with resin, with 19 different isolations by both methods. There were 2 samples from which virus could not be recovered by the resin, as compared to 6 failures by the routine procedures. Virus could not be recovered on a second attempt on these 6 samples by the routine method. In a second group, 73 samples which had previously been found negative by the routine method were retested. Of these, there were 4 virus isolations by the routine method and 10 by the resin method. In both experiments, there were a total of 12 samples from which virus was isolated by resin treatment but not by the routine method on 2 different tests.

Recently Kelly(7) has reported that resin treatment improved detection of Coxsackie virus in sewage. The evidence presented in Table II shows that the number of virus isolations was increased by the use of a cation resin. Although the volume of eluate used was one-half the original amount of sample added to the resin, the degree of virus concentration is slight, if any. A possible explanation for the increased number of virus recoveries by resin treatment may be that by such a procedure, the throat sample is cleared of virus inhibitors, either specific antibodies or non-specific substances present in the respiratory tract. The technics involved in the use of ion exchange resins are simple and may be useful in improving the efficiency of virus isolation.

Summary. Early passage, egg-adapted Type A influenza virus is completely adsorbed on a cation exchange resin, and nearly all of the adsorbed virus can be eluted as infective particles. Influenza A virus was recovered from human throat washings and swabs more frequently with the use of resin than without it.

---

1. Muller, R. H., PROC. SOC. EXP. BIOL. AND MED., 1950, v73, 239.

2. Lo Grippo, G. A., *ibid.*, 1950, v74, 208.

3. Muller, R. H., and Rose, H. M., *ibid.*, 1952, v80, 27.

4. Puck, T., and Sagik, B., *J. Exp. Med.*, 1953, v97, 807.

5. Reed, L. J., and Muench, H., *Am. J. Hyg.*, 1938, v27, 493.

6. Philip, R. N., Bell, J. A., Davis, D. J., Beem, M. O., Beigelman, P. M., *Am. J. Pub. Health*, 1954, v44, 34.

7. Kelly, S. M., *ibid.*, 1953, v43, 1532.

Received April 2, 1954. P.S.E.B.M., 1954, v85.

Infertility Induced in Mice by a Single Injection of Testosterone Propionate.* (20989)

C. A. Barraclough† and J. H. Leathem.

From the Bureau of Biological Research and Department of Zoology, Rutgers University, New Brunswick, N. J.

The influence of androgens on the female reproductive tract has received considerable attention but comparatively little is known about the effects of these steroids on fertility. In general, reproductive performance in female rats is adversely affected by testosterone propionate. Daily administration of testosterone propionate to female rats from birth to 44 or 175 days of age induced sterility(1). Furthermore, if the androgen was given on alternate days starting at 6 days of age and continued until the rat was 38 days old, mating attempts failed as long as 10 weeks later (2). Adult female rats treated with 2 mg of testosterone propionate daily for 4 weeks failed to exhibit vaginal plugs although caged with males. A month after cessation of treatment, remating was attempted with 98% success(3). It would appear that the age at which androgen injections are started is an important factor in the establishment of a permanent or temporary sterility. To further explore this premise, the influence of single injections of testosterone propionate given to mice at definite prepuberal ages was tested for an effect on fertility.

Methods. Swiss albino mice raised in our laboratory were used in this study. The mice were maintained on Rockland complete mouse diet pellets. The diet was supplemented by a mixture of cod liver oil and Mazola oil on bread twice weekly and by lettuce once each week. Female mice of 5, 10 and 20 days of age were given a single subcutaneous injection of 1.0 mg of testosterone propionate (Oreton, Schering)‡ in oil. The single injection permitted a minimum of disturbance to the nursing animals which were kept with their mothers until weaning. The mice selected were of such number as to enable an approximately equal distribution of littermates between control and injected groups. Fertility was tested when the mice reached 90 days of age by placing the treated and control mice from one litter with a male. The time after admitting the male until littering was recorded as well as the litter size, sex distribution and the number and weight of young carried to weaning at 22 days of age. Litters were reduced to 6 animals at 3 days after birth to permit more uniform growth.

Results. The single injection of testosterone propionate to 5-day-old mice sharply curtailed fertility. These mice were kept with proven males for 100 days (now 6 months old) without obtaining litters from 14 animals. In contrast, all littermate control mice delivered litters in a month. Two mice destroyed their litters but the others carried their young to weaning. Lactation was subnormal in this control group, however, as indicated by the weaning weight of the pups (Table I).

Androgen administration at 10 days of age had less effect on fertility than when given at 5 days of age as only 4 of 12 mice failed to produce litters. Those mice which were fertile, dropped litters at essentially the same time as controls, had a normal number of pups per litter and exhibited good lactation.

Testosterone propionate injections at 20 days of age failed to diminish fertility (Table I).

Ovaries of infertile mice revealed relatively few follicles, no corpora lutea and a marked concentration of sudanophilia in the prominent stroma. Furthermore, follicular atresia was increased by androgen in 5-day-old mice examined at various times after injection but had little effect on older mice(4).

Estrogen will also induce infertility in mice

---

* Supported by a grant from E. R. Squibb & Sons.

† Present address: Department of Anatomy, University of California at Los Angeles.

‡ Grateful acknowledgement is made to Dr. E. Hendersen, Schering Corp. for the testosterone propionate.

TABLE I. Influence of Age and Testosterone Propionate (1 mg) on Reproductive Performance of Prepuberal Mice.

Treatment	No. mice	No. preg.	Days in breeding to 1st litter, avg (range)	Avg No./litter	Avg* weaning wt (g)
Control	14	14	31.6 (22-40)	6.6	5.9
T.p. at 5 days	14	0	Infertile	—	—
Control	13	13	33.3 (22-46)	6.5	10.0
T.p. at 10 days	12	8	37.0 (28-55)	7.0	9.7
Control	14	14	39.4 (22-60)	7.1	11.7
T.p. at 20 days	16	15	35.7 (22-71)	7.8	10.9

* Per individual at 22 days.

and the prepuberal ages appear to be the more sensitive periods(5,6).

Summary. Mice 5, 10 and 20 days of age were given a single 1 mg injection of testosterone propionate. Fertility was tested when these mice were 3 months old and compared with littermate controls. No litters were obtained with mice treated at 5 days of age despite 100 days in breeding. Fertility of mice treated when 20 days old, however, was not decreased whereas mice treated at 10 days of age assumed an intermediate position.

1. Shay, H., Gershon-Cohen, J., Paschkis, K. E., and Fels, S. S., *Endocrinology,* 1939, v25, 933.
2. Bradbury, J. T., *ibid.,* 1941, v28, 191.
3. Huffman, J. W., *ibid.,* 1941, v29, 77.
4. Barraclough, C. A., *Anat. Rec.,* 1954, v118, 281.
5. Emmens, C. W., *J. Endocrinol.,* 1953, v9, 204.
6. Merklin, R. J., *Endocrinol.,* 1953, v53, 342.

Received April 2, 1954. P.S.E.B.M., 1954, v85.

Distribution of Succinic Dehydrogenase and Hyaluronidase in Adult Rat Testis.* (20990)

ROGER L. GREIF. (Introduced by R. F. Pitts.)

From the Hospital of the Rockefeller Institute for Medical Research, New York City, and the Department of Physiology, Cornell University Medical College, New York City.

A study of testicular hyaluronidase has revealed, in confirmation of the work of Perlman *et al.*(1) that freezing and thawing and sterile autolysis of homogenates of testicular tissue make possible the extraction of large amounts of this enzyme. These steps were therefore incorporated into a reproducible method for the measurement of the hyaluronidase content of rat testes(2). The relatively low activity found in extracts of fresh unfrozen and unautolyzed tissue suggested that hyaluronidase might be localized within a structure which was altered by freezing and autolysis.

Arnesen, Buxton, and Dulaney(3) concluded from differential centrifugation experiments with homogenates of mouse testis suspended in hypertonic sucrose solution (.88 Molar) that hyaluronidase activity was localized in the so-called "soluble" fraction. The fractions obtained by these workers were tested neither for activity of an enzyme of known localization, nor for nitrogen content. In the present investigation the determination of succinic dehydrogenase activity and of nitrogen content have been employed as biochemical indications of the effectiveness of the separation procedure(4). Optimum conditions for the measurement of the hyaluronidase content of testicular fractions are still being sought, hence the results to be reported are of a preliminary nature.

Methods. Adult albino rats were killed by means of a blow on the head, the testes were

---

* This investigation was supported in part by the Helen Hay Whitney Foundation.

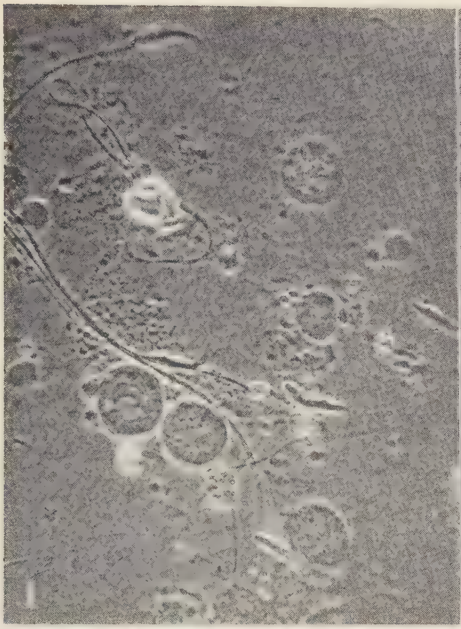


FIG. 1. Phase microscopic appearance of suspension of testicular homogenate in .25 M sucrose ("Total" fraction) × 1200.

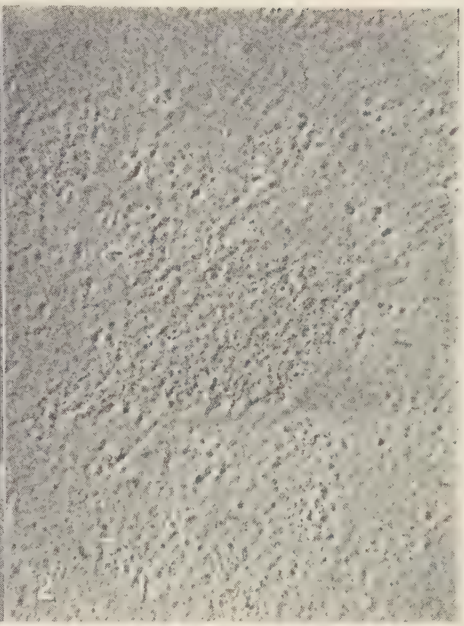


FIG. 2. Phase microscopic appearance of "granular" fraction × 1200.

removed immediately, stripped of *Tunicae albugineae,* and the remaining pulp weighed. All subsequent fractionation operations were carried out in a cold room at 0-4°C. The pulp was ground by hand in an all-glass tissue grinder, after which it was suspended in cold .25 M sucrose solution in a concentration of 0.2 g wet weight per ml of suspension. Fig. 1 is a phase contrast photomicrograph of such a preparation, and is seen to contain unbroken cells, nucleii, intact spermatozoa, sperm heads and tails, large cytoplasmic masses and granular material.[†] This uncentrifuged suspension will hereafter be referred to as "total". A portion of this suspension was centrifuged at 600 x g (International Centrifuge size 1) for 45 minutes. The sediment from this step, hereafter referred to as the "nuclear" fraction, on phase microscopic examination resembled Fig. 1. The supernatant fluid from the first centrifugation contained granular material. This fluid was subjected to centrifugation for one hour at 8500 x g (Servall S S angle centrifuge). A sediment was obtained which was light tan in color and had transparent edges. Fig. 2 illustrates the phase microscopic appearance of a suspension of this "granular" fraction. The supernatant fluid from the latter centrifugation appeared clear or slightly turbid, and on microscopic examination contained few visible structures other than fat globules. It will hereafter be called the "soluble" fraction, although the experience of others with differential centrifugation of liver homogenates suggests that microsomes are present in this preparation(4). To obtain a better yield of granular fraction the short procedure outlined above was modified as follows: the nuclear fraction was resuspended in fresh sucrose solution with the use of the tissue grinder, following which it was recentrifuged at 600 x g for 45 minutes. The supernatant liquids from 3 such nuclear washings were pooled with the supernatant fluid from the first centrifugation, and this pool was centrifuged at 8500 x g for one hour to obtain the granular fraction. Succinic dehydrogenase activity was measured on 2 different sized aliquots of the same specimen by

---

[†] To avoid blurring due to Brownian movement during exposure, the granular material in these phase photomicrographs was caused to aggregate by the addition of Janus Green B to the suspension.

TABLE I. Mean Succinic Dehydrogenase Activity and Nitrogen Content of Rat Testicular Fractions. (Seven separate fractionations in which the nuclear fraction is unwashed.)

Sample	O_2 uptake/hr/g testis wet wt (μl)	mg N/g testis wet wt	μl O_2 taken up/hr/mg of N (specific activity)
Total	1948±235*	17.35± .68	112±12
Nuclei	1133±231	7.93± .98	143±24
Granules	687±121	2.53± .51	273±32
Soluble	<10	6.21±1.28	
% of total recovered in fractions	93.6±6.5	95.6 ±7.6	

* Stand. dev. about the mean.

the manometric technic of Schneider and Potter(5). The cytochrome C required for this method was obtained commercially.‡ Hyaluronidase and nitrogen determinations were made as previously described(2).

Results. Succinic dehydrogenase distribution. Table I shows the succinic dehydrogenase activity and the nitrogen content of testicular fractions obtained with the short procedure. Activities are referred to both wet weight of tissue and nitrogen content, and represent the mean value and standard deviation about the mean for 7 separate fractionations. It will be seen that the highest specific succinic dehydrogenase activity is present in the granular fraction, but that only 35% of the total amount of enzyme is present in that fraction.

The results obtained after use of the modified technic which involves 3 washings of the nuclear fraction are recorded in Table II. These data, compiled from 10 separate fractionations, show that the succinic dehydrogenase content of the nuclear fraction can be reduced by this washing procedure so that a larger proportion of the total amount of enzyme can be found in the granular fraction. Some loss of activity has resulted from the additional manipulations. The specific activity of the granular fraction remains higher than that of the nuclear and of the total fractions.

TABLE II. Mean Succinic Dehydrogenase Activity and Nitrogen Content of Rat Testicular Fractions. (Ten separate fractionations in which the nuclear fraction was washed 3 times.)

Sample	O_2 uptake/hr/g testis wet wt (μl)	mg N/g testis wet wt	μl O_2 taken up/hr/mg of N (specific activity)
Total	1879±146*	17.75±.78	106± 8
Nuclei	564±174	6.05±.93	93±24
Granules	1025±197	3.39±.40	303±48
Soluble	<10	6.69±.76	
% of total recovered	84.7±5.8	91.6 ±5.1	

* Stand. dev. about the mean.

After 6 washings, the specific succinic dehydrogenase activity of the nuclear fraction can be reduced to unmeasurable levels. Since this activity is based upon nitrogen content, the results of repeated washing cannot be explained on the basis of the large loss of nuclear material which occurs through fragmentation and distribution to other fractions.

The granular fraction probably includes mitochondria and microsomes as well as some nuclear fragments, and contains material which stains supravitally with Janus Green B. In 3 experiments this fraction was repeatedly resuspended in sucrose solution and centrifuged for 30 minutes at 25000 x g. In Table III will be found the specific succinic dehydrogenase activity of the fractions obtained as indicated in column 1. In contrast to the results of washing the nuclear fraction, the specific activity of the granular fraction remains constant or actually increases as inactive nitrogenous material is removed by washing.

Hyaluronidase distribution. Sterile autolysis at 37°C under toluene for 48 hours was required to obtain high hyaluronidase activity from the individual fractions. A large number of variables are introduced with this autolysis step, and to date it has not been possible to recover in the fractions all the activity present in the uncentrifuged preparation. The results of the hyaluronidase assay are therefore indicated in Table III in an approximate way only. It is evident that hyaluronidase activity persists in the washed granular fraction, and the data suggest also that the specific activity of the granular fraction is higher than that of the total preparation. The specific activity of the nuclear fraction can be reduced to zero

---

‡ Sigma Chemical Company, St. Louis, Mo.

TABLE III. Effect of Repeated Washing with .25 M Sucrose upon Specific Succinic Dehydrogenase and Hyaluronidase Activity. (3 experiments arranged in columns a, b, and c respectively.)

Sample	Specific succinic dehydrogenase activity (μl O_2 uptake/mg N/hr)			Estimated hyaluronidase activity/mg N		
	(a)	(b)	(c)	(a)	(b)	(c)
Total	112	130	97	2+	2+	3+
Granules (unwashed)	306	368	144	—	3+	3+
Granules (washed 6×)	385	405	277	4+	4+	3+

by repeated washing. No more than traces of activity have been found in the soluble fraction.

Discussion. The large number of cell types present in the mature rat testis make this tissue far less satisfactory than liver for cell fractionation studies(6). The heterogeneous nature of the granular fraction isolated from liver homogenates(7) is even more striking in the case of the testis because the granules doubtless come from ruptured cells of the germinal epithelium, interstitial cells, and cells of supporting tissue. Thus far no attempt has been made to subdivide the granular fraction into mitochondria and microsomes. It seems evident, despite technical difficulties, that hyaluronidase activity can be found in the granular fraction of the rat testis and that it persists even after repeated washing. Should further experimentation confirm this observation, it is possible that the role of hyaluronidase, at present conjectural, in the fertilization process might be assigned to the midpiece of the spermatozoon, which has been observed to contain mitochondrial material and oxidase activity(8).

A number of mitochondria-linked enzymes, among them an acid phosphatase(9) and adenosine triphosphatase(10) have shown low activity in mitochondria immediately following their isolation from sucrose solution. The enzyme activity of these mitochondria increases following water or detergent extraction or simple aging. A similar process, presumably alteration of the mitochondrial membrane, may be responsible for the release of hyaluronidase from testicular homogenates after freezing and thawing and autolysis. Microscopic examination of tissue so treated reveals striking structural alteration. The physiological significance of enzymes which require drastic treatment for release from tissue remains obscure.

Summary. The distribution of succinic dehydrogenase in the testis of the adult albino rat has been studied by means of differential centrifugation in .25 M sucrose solution. The highest specific activity has been found in a granular fraction, and preliminary evidence is presented to indicate that hyaluronidase activity is also associated with this fraction.

The author is indebted to Dr. George Palade for assistance with cytological examinations and to Mrs. Elnora W. Van Winkle for technical assistance.

---

1. Perlman, P. L., Leonard, S. L., and Kurzrok, R., *Endocrinol.*, 1948, v42, 26.

2. Greif, R. L., *J. Biol. Chem.*, 1954, v206, 381.

3. Arnesen, K., Buxton, L., and Dulaney, A. D., PROC. SOC. EXP. BIOL. AND MED., 1949, v71, 264.

4. Schneider, W. C., *J. Biol. Chem.*, 1948, v176, 259.

5. Schneider, W. C., and Potter, V. R., *ibid.*, 1943, v149, 217.

6. Schneider, W. C., and Hogeboom, G. H., *Cancer Res.*, 1951, v11, 1.

7. Novikoff, A., Podber, E., Ryan, J., and Noe, E., *J. Hist. and Cytochem.*, 1953, v1, 27.

8. Elftman, H., in Engle, E. T., *Studies on Testis and Ovary Eggs and Sperm*, Charles C. Thomas Co., Springfield, Ill., 1952, p35.

9. Berthet, J., and de Duve, C., *Biochem. J.*, 1951, v50, 174.

10. Kielley, W. W., and Kielley, R. K., *J. Biol. Chem.*, 1951, v191, 485.

Received April 5, 1954. P.S.E.B.M., 1954, v85.

Effect of Diet and Trace Minerals on Erythrocyte Fragility and x-Irradiation Mortality in Rats.* (20991)

Marguerite A. Constant and Paul H. Phillips.

From the Department of Biochemistry, College of Agriculture, University of Wisconsin, Madison.

Several studies have shown that hemorrhage or loss of red blood cells into the lymphatics frequently were responsible for the severe anemia seen post-irradiation(1). Evidence of increased red cell destruction has also been obtained(2). Chanutin *et al.*(3) observed that whole body irradiation (500 r) caused an increased red cell destruction following phenylhydrazine treatment. Erythrocyte fragility determinations were not made. Goldschmidt *et al.*(4) found that the fragility of rat erythrocytes reached a maximum 12 hours post-irradiation (600 r) but had returned to normal in 24 to 48 hours. Recent studies by Harman(5) have shown that the erythrocytes of rats fed stock diet were more fragile than the erythrocytes of rats fed a semi-purified diet. It appeared desirable, therefore, to study the effect of diet, starvation or whole body irradiation on erythrocyte fragility in order to correlate, if possible, the ease of destruction of erythrocytes and the lethality of whole body irradiation.

Experimental. Weanling male rats of the Sprague-Dawley strain received food and water *ad libitum* and were given 2 drops of haliver oil weekly. Three basal diets were used in these experiments. Diet 1 was Rockland guinea pig diet which contained 17% protein and 2.5% fat (minimum values). Diet 2 was composed of 45% corn flakes, 20% wheat flakes, 12% soybean oil meal, 12% meat scraps, 5% alfalfa, 3% brewer's yeast (dried), 3% fish solubles and 0.01% irradiated yeast (22% protein, 3% fat). Diet 3 was a semi-synthetic diet composed of 18% crude casein, 4% Salts IV, 68% sucrose, 10% corn oil or Crisco and B vitamins. The B vitamin supplement contributed, in mg/kg of ration, thiamine HCl, 3.8; pyridoxine HCl, 3.8; riboflavin, 4.5; niacin, 38.0; Ca-pantothenate, 30.0; inositol, 100; biotin, 0.1; folic acid, 0.2; and 1.0 g choline. The trace mineral supplements contained 1.0 to 0.5 mg Co, 0 to 2.0 mg Cu and 0 to 7.5 mg Mn per 10 g of ration depending upon the experiment as indicated. Samples of blood were taken from the heart under nembutal anesthesia (6 mg/100 g of body weight, intraperitoneally). Erythrocyte fragilities were determined by the method of Harman(5). Determinations on blood samples from an animal in the control and each experimental group were made simultaneously. The concentration of the hypotonic solutions were graded stepwise by 0.03% from 0.35% (Exp. 1) or 0.02% from 0.36% (Exp. 2 and 3) to 0.50%. Since all solutions in these ranges gave similar results data are reported only for representative concentrations. The amount of *whole body irradiation* administered to animals in each experiment was 700 r delivered from a 200 KV Westinghouse therapeutic machine at the rate of 20 r/minute, 175 KV, 10 ma, 1 mm Al, 0.2 mm Cu, HVL 0.83 mm Cu, 40 cm from target.

Results. The first series of experiments was a study of the effect of starvation and trace minerals on erythrocyte fragility. Diets 1 (natural), 3 (synthetic) and 3 plus 10 mg % Co, 2 mg % Cu, and 5 mg % Mn were used. Although fragility determinations were not reported by Harman(5) until after 3 months of feeding, it was apparent from our data that the difference in osmotic fragility of erythrocytes due to diet was evident at 35 days. By the 41st day (Table I) the rbc's of the majority of animals fed diet 3 were 50% less fragile. Starvation periods of 8 to 24 hours did not affect the degree of fragility. The hemoglobin values at this time were 13.8 ± 0.8, 13.4 ± 0.3, and 17.3 ± 0.8 g for animals fed diets 1, 3 and 3 plus trace minerals respectively. Fragility data obtained 24 and 120 hours post irradiation showed that the greater osmotic stability of rbc's of rats

---

* Published with the approval of the Director of the Wisconsin Agric. Exp. Station. Supported in part by grants from the U. S. Atomic Energy Commission.

TABLE I. Effect of Diet and Starvation on Erythrocyte Fragility and Mortality from X-Irradiation.

	% hemolysis in 0.41% NaCl			% hemolysis in alloxan§		Survivors
Day	41*	46†	50‡	46	50	+20
Hr starved	0–24	24	120	24	120	—
Diet No.						
1 (natural)	74 ± 7	97 ± 5‖	82 ± 11	51 ± 7	57 ± 4	
1 + irradiation		93 ± 5	77 ± 20	54 ± 7	54 ± 11	1
3 (synthetic)	35 ± 12	60 ± 11	45 ± 9	16 ± 7	29 ± 5	
3 + irradiation		62 ± 12	28 ± 9	13 ± 9	27 ± 6	3
3 + Co, Cu, Mn	29 ± 7	53 ± 6	62 ± 16	9 ± 4	27 ± 6	
3 + *idem* and irradiation		49 ± 11	50 ± 22	10 ± 8	21 ± 4	3

* 12 rats/series.

† 24 hr post-irradiation; 4 rats/control, 8/irradiated series.

‡ 4 rats each series, 4 for survival.

§ Increased hemolysis in 25 min. in alloxan (10 mg % in .50% NaCl) compared to 30 min. in .50% NaCl.

‖ $\pm\sqrt{\frac{\Sigma x^2}{N}}$.

fed diets 3 and 3 plus Co, Cu and Mn was still evident. Prolonged starvation (120 hours) did not alter the fragility. The rbc's of irradiated animals were not more fragile than the rbc's of control animals. An explanation for the general increase in rbc fragility seen in all groups cannot be given at this time. Temperature and pH among other factors have been shown by Parpart *et al.*(6) to affect fragility. Venous rbc's were observed to be more fragile than arterial rbc's from the same animal but that difference was not sufficient in magnitude to invalidate the differences caused by dietary treatment. The poor survival rate of animals fed diet 1 indicated that animals with more fragile rbc's may be more susceptible to irradiation effects.

A second series of experiments was made to determine whether an increased fragility of rbc's and poor survival rate of animals fed a different natural diet would occur in comparison to results with the synthetic diet. The effect of diets 2 (natural) and 2 plus trace minerals was compared with that of diets 3 and 3 plus trace minerals. The level of Co, Cu and Mn used initially in this experiment was 10 mg %, 4 mg % and 15 mg % respectively. Since it was observed that the

TABLE II. Effect of Diet, Dietary Trace Minerals and Irradiation on Erythrocyte Fragility and Mortality.

	% hemolysis in 0.40% NaCl						Survivors	
Day	18*	29*	39	45	54	60†		
Hr starved	0	0	24–48	24–48	0	24	+15	+30
Diet No.								
2 (natural)	51 ± 13	35 ± 24	69 ± 11	56 ± 17	62 ± 13	78 ± 8	6	4
2 + Co, Cu, Mn	44 ± 16	31 ± 14	45 ± 9	33 ± 9	30 ± 8	48 ± 10	1	1
3 (synthetic)	31 ± 12	9 ± 4	33 ± 9	19 ± 9	18 ± 6	28 ± 9	7	5
3 + *idem*	34 ± 11	16 ± 8	44 ± 10	32 ± 10	35 ± 9	41 ± 9	5	4
	Increased hemolysis in alloxan solution							
2	39 ± 6	31 ± 8	25 ± 5	38 ± 12	28 ± 8	39 ± 13		
2 + *idem*	35 ± 8	28 ± 9	17 ± 5	19 ± 6	9 ± 3	18 ± 5		
3	21 ± 6	19 ± 10	7 ± 2	12 ± 5	6 ± 2	12 ± 6		
3 + *idem*	23 ± 3	13 ± 6	10 ± 3	15 ± 4	10 ± 3	14 ± 3		

* 6 rats/series (0.41% NaCl) day 18–29, 8 rats/series day 39 to +30.

† 24 hr post-irradiation.

TABLE III. Effect of Dietary Cobalt on Erythrocyte Fragility and Mortality in the Rat. 10 rats/series.

Diet No.	Concentration of solutions			Alloxan,	No. surviving irrad.†	
	.36%	.38%	.40%	% hemolysis*	Days	
	% hemolysis				15	30
Exp. 3A—0 hr starvation; day 26						
1	94 ± 5	86 ± 6	70 ± 12	42 ± 8		
1 + Co	88 ± 6	83 ± 8	73 ± 13	46 ± 6		
2	70 ± 8	47 ± 13	25 ± 11	19 ± 7		
2 + Co	48 ± 16	36 ± 12	23 ± 9	13 ± 7		
3	60 ± 16	42 ± 15	21 ± 12	15 ± 8		
3 + Co	77 ± 11	62 ± 16	43 ± 16	26 ± 10		
Exp. 3B—0 hr starvation; day 46						
1	90 ± 6	77 ± 10	56 ± 14	35 ± 8	4	3
1 + Co	70 ± 11	58 ± 12	43 ± 11	22 ± 7	3	3
2	88 ± 7	78 ± 8	56 ± 13	28 ± 8	7	7
2 + Co	39 ± 10	27 ± 8	18 ± 6	6 ± 2	5	3
3	71 ± 6	52 ± 7	28 ± 6	12 ± 5	8	5
3 + Co	64 ± 12	51 ± 13	31 ± 9	13 ± 4	1	1

* Increased hemolysis in 25 min. in alloxan (10 mg % in 0.50% NaCl) compared to 30 min. in 0.50% NaCl.

† Separate groups of 8 animals each were used for mortality study.

food became rancid after a 48 hour exposure to air, the level of trace minerals was halved and the corn oil was replaced by Crisco at the end of the first week. The cobalt level only was increased to 10 mg % on the 33rd day. No rancidity was detected. A decreased fragility of rbc's of rats fed diet 3 was again observed and was measurable on the 29th day (Table II). Starvation periods up to 48 hours were again found to have no effect. The data obtained on the 39th day showed that the fragility of rbc's of rats fed diet 2 (natural) plus Co, Cu and Mn had decreased and was comparable to that of rbc's of animals fed the synthetic diets, a fact still evident 24 hours post-irradiation. The decreased fragility of rbc's of animals fed the supplemented diet 2, diet 3 and the supplemented diet 3 compared to that of animals fed diet 2 only was also evident in the alloxan solutions. The mortality data in Table II showed that 700 r was greater than an LD_{50} dose only for animals fed the supplemented diet 2.

A third experiment was made to determine the effect of dietary cobalt upon erythrocyte fragility and mortality. Cobalt was fed at a level of 10 mg %. The diets fed were 1) diet 1, 2) diet 1 + Co, 3) diet 2, 4) diet 2 + Co, 5) diet 3, and 6) diet 3 + Co. The level of fat in the synthetic diets (lots 5 and 6) was reduced to 5% in order to have this dietary constituent comparable in all diets. Hemolysis data (Table III) obtained on the 26th day showed that the erythrocyte fragility of animals fed diet 1 was greater than that of animals fed diets 2 or 3. No effect of cobalt on erythrocyte fragility was seen in any of the groups. By the 46th day the fragility of rbc's of rats fed diet 2 had increased and was comparable to that of animals fed diet 1. A marked reduction in the fragility of rbc's of animals fed the cobalt supplemented diet 2 was observed. The supplementation of diet 1 with Co had no effect on erythrocyte fragility by the 46th day. Although the fragility of rbc's of rats fed diet 3 with 5% fat was somewhat higher than observed previously, Co supplementation was without effect on erythrocyte fragility.

The mortality data in Table III showed that 700 r was again more than an LD_{50} dose for animals fed diet 1, diet 1 plus Co, or diet 2 plus Co and was less than an LD_{50} dose for animals fed diet 2 only or diet 3 only. The rapid mortality rate of animals fed the supplemented synthetic diet was unexpected in view of the findings of the first two experiments. Since the only difference in the synthetic diets used in this and the previous experiments was the dietary level of fat, the data indicate that the decreased level of dietary fat in the cobalt supplemented synthetic diet might have been responsible for the increased mortality rate.

Discussion. The observation reported by Harman(5) that rbc's of rats fed a natural diet were more fragile than the rbc's of rats fed a synthetic diet was confirmed and extended by these studies.

In addition it was found that the supplementation of diet 2 (natural) with Co, Cu and Mn or cobalt only caused a marked reduction in erythrocyte fragility. A reduction in fragility of rbc's from animals fed the cobalt supplemented diets 1 (natural) or 3 (synthetic) was not observed. These data showed that the effect of trace minerals in reducing the erythrocyte fragility in the tests referred to earlier in this report was due to the dietary cobalt. These data also showed that the action of cobalt in reducing erythrocyte fragility was pronounced only and associated with the feeding of diet 2.

Because of the effect of cobalt on cellular respiration and the production of polycythemia, Parr *et al.*(7) fed a cobalt supplemented diet to mice for 8 days prior and 15 days following irradiation. This supplement, which was prepared by immersing pellets in a 2% $CoCl_2$ solution, afforded protection to the treated animals. Our studies with rats indicate that prolonged treatment of animals before and after irradiation with cobalt may be detrimental.

A summary analysis of the mortality data obtained in these experiments showed that 700 r was an LD_{67} for animals fed diet 1 (7/12 dead on 15th day); an LD_{63} for animals fed the supplemented diet 1 (5/8 dead on 15th day); an LD_{75} for animals fed the supplemented diet 2 (10/16 dead on 15th day); and LD_{31-38} for animals fed diets 2 only or 3 only. These data indicate that the pre-irradiation fragility of erythrocytes was of less importance in determining the lethality of irradiation than the type and adequacy of the diet.

In the second series of experiments the incisors of all animals fed the supplemented diet 2 were observed to be almost completely depigmented in approximately 2 to 3 weeks. The incisors of all other animals were normal. A similar observation was made in the third experiment and included several of the animals fed the supplemented diet 3. The incisors of animals fed diet 1, diet 1 plus Co, diet 2 and diet 3 were normal. Additional precautions were made to assure adequate vitamin A intake by doubling the haliver oil supplement. This procedure was without effect. Rose and Gyorgy(8) have reported an increased fragility of rbc's of rats mildly deficient in vit. E. However, a decreased fragility of rbc's of animals fed the supplemented diet 2 was observed. These data indicate that the bleaching effect caused by the supplement was probably not due to avitaminosis A or avitaminosis E. An explanation for this bleaching effect cannot be given at this time.

Summary. The data indicate that the fragility of erythrocytes pre-irradiation was of less importance in determining the lethality of irradiation than the type and adequacy of the diet. The three basal diets used in these studies supported growth equally well; however, animals fed diet 1 (natural) succumbed to the effects of irradiation more rapidly than did animals fed diet 2 (natural) (rbc's equally fragile) or those fed diet 3 (synthetic) (rbc's less fragile). Dietary trace mineral supplementation was observed to reduce the erythrocyte fragility. This effect which was traced to the cobalt of the supplement was pronounced and was associated with the feeding of one of the natural diets (diet 2). Although the rbc's of animals fed the supplemented diet 2 were less fragile, these animals showed an earlier and more rapid mortality rate than did animals fed the basal diet. The data from the present studies indicate that long term feeding of polycythemic levels of cobalt increased the mortality rate of animals given whole body irradiation.

---

1. Cronkite, E. P., and Brecher, G., *Ann. Rev. Med.*, 1953, v3, 193.

2. Davis, R. W., Dole, N., Izzo, M. S., and Young, L. E., *J. Lab. Clin. Med.*, 1950, v35, 528.

3. Chanutin, A., Lentz, E. A., and Ludewig, S., *Am. J. Physiol.*, 1953, v173, 474.

4. Goldschmidt, L., Rosenthal, R. L., Bond, V. P.,

and Fishler, M. C., *ibid.*, 1951, v164, 202.

5. Harman, J. W., PROC. SOC. EXP. BIOL. AND MED., 1952, v79, 301.

6. Parpart, A. P., Lovenz, P. B., Parpart, E. R., Gregg, J. R., and Chase, A. M., *J. Clin. Invest.*, 1947, v26, 636.

7. Parr, W., O'Neill, T., and Krebs, A., *Science*, 1953, v117, 155.

8. Rose, C. S., and Gyorgy, P., PROC. SOC. EXP. BIOL. AND MED., 1950, v47, 411.

Received January 29, 1954. P.S.E.B.M., 1954, v85.

Prolongation of the Lag Phase of Irradiated *Escherichia coli*. (20992)

E. BEUTLER, MELBA J. ROBSON, AND L. O. JACOBSON.

From the Department of Medicine and Argonne Cancer Research Hospital, The University of Chicago, Chicago, Ill.

Although the lethal effect of ionizing radiations on bacteria has been the subject of repeated and extensive study(1), the recovery of damaged organisms has received relatively little attention. In the course of attempts to modify the lethal effect of X rays on bacteria, we have observed a delay in the onset of multiplication of the *surviving* organisms. Because recovery from radiation injury in higher animals may well be dependent on the resumption of growth by damaged cells rather than on the revival of "killed" cells, we believed that this aspect of the problem deserved further investigation.

Materials and methods. Bacteria. A culture of *Escherichia coli*,* serially passed on blood agar plates and stored in a refrigerator, was used throughout these studies. Unless otherwise stated, all cultures were incubated aerobically at 37°C. Pour plates were made by adding one cc of culture, diluted to produce approximately 50 to 200 colonies, to 8 cc of melted tryptocase soy agar† at 40 to 44°C. Colony counts were made after an 18-hour incubation period. Reincubation of some plates from both irradiated and non-irradiated cultures for additional 24- and 48-hour periods failed to give any increase in colony counts. *Irradiation.* Cultures were irradiated in Evelyn spectrophotometer tubes with 250-kvp X rays at 15 ma with .25-mm Al filtration. The tubes were held in an ice water bath by a modification of the apparatus described by Stapleton *et al.*(2). Exposure rates, calibrated with a Victoreen thimble chamber inside of an Evelyn tube, varied from 425 to 480 r per minute. In each experiment, a single overnight culture of *E. coli* in tryptocase soy broth served as the source of both control and irradiated bacteria. Except where otherwise indicated, the original culture was diluted sufficiently in tryptocase soy broth prior to irradiation so that pour plates could be made from irradiated and control cultures without further dilution. Because the survival ratio of the strain of *E. coli* employed was approximately one in 2500 at 30,000 r, the non-irradiated cultures were diluted 10^3- and 10^4-fold further than the cultures receiving this exposure. All cultures were kept in ice water baths from the time of dilution, throughout irradiation, and until after the initial pour plates had been made after irradiation. After pour plates had been prepared from irradiated and control cultures, they were placed into a 42°C water bath and warmed for 5 minutes prior to transfer to a 37°C incubator. The control and irradiated cultures were handled identically in all respects throughout: equal volumes were moved in and out of the water baths and incubator in equal-sized tubes.

Results. The growth of irradiated and non-irradiated control cultures was studied repeatedly. Typical growth curves are shown in Fig. 1. Cultures that had been X-irradiated with 30000 r consistently showed a ¾- to 1½-hour delay in the onset of the phase of logarithmic

---

* Isolated from a patient and supplied to us through the kindness of Miss Dorcas Fasan, Department of Pediatrics, University of Chicago.

† Baltimore Biological Laboratory, Baltimore, Md.

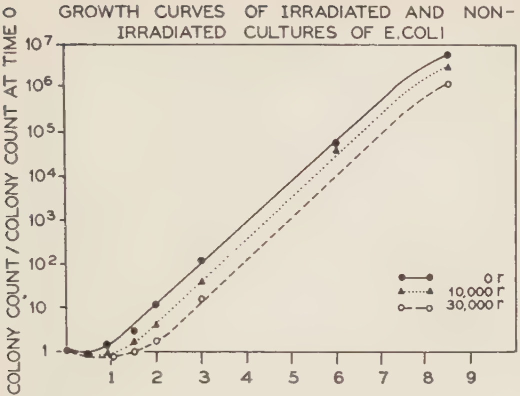


FIG. 1.

growth when compared with unirradiated controls. With 10000 r, the delay was shorter. A slight decrease in the colony count was observed frequently during the lag phase. The logarithmic growth rate was the same for irradiated and control cultures.

Because it appeared likely that the prolongation of the lag phase of irradiated cultures might be due to toxic changes induced in the culture medium, a more concentrated culture was irradiated and subsequently diluted 100-fold into fresh medium. The growth curve of irradiated bacteria thus transplanted into non-irradiated medium was identical with that plotted for growth in an irradiated medium.

Prolongation of the lag phase was increased to 2½ hours and the rate of growth in the logarithmic growth phase was considerably reduced when incubation subsequent to irradiation was carried out at 24°C.

The effect of storage at –2°C and 7°C on the lag phase of irradiated and control cultures was also studied. Because the colony counts of cultures stored at these temperatures decreased progressively, undiluted cultures were irradiated and diluted as necessary at the end of the storage period. Colony counts were made before and 2 hours after re-incubation of the stored culture. Even after a 27-day cold storage period there was no significant multiplication of irradiated cultures upon incubation. However, the non-irradiated cultures multiplied at a reduced rate after cold storage for as little as one week. This suggests that cold storage alone induces a prolongation of the lag phase. These 2 means of prolongation proved to be additive in cultures that had been stored at cold temperatures for several weeks and then X-irradiated.

The modifying effect of various substances on the X-ray-induced prolongation of the lag phase was studied to assay materials for radiation-recovery potency. In each experiment, the simultaneous growth of non-irradiated control cultures and irradiated cultures was compared. Test substances were added after irradiation, but prior to incubation. The growth at the end of 2 hours was selected as a convenient endpoint for screening purposes, colony counts being made, in general, only before and 2 hours after incubation. Media containing 7.5% freshly drawn mouse blood; 15% rabbit serum, which had been heated to 56°C for ½ hour; fresh 0.5% cysteine solution, added before as well as after irradiation; or the filtrate of an actively-growing non-irradiated *E. coli* culture all failed to reverse the radiation-induced lag phase. The addition of fresh spleen, liver, or kidney mash from unirradiated mice consistently altered the growth curve of irradiated organisms in the direction of normal. However, the addition of these tissues also increased the rate of growth of non-irradiated cultures. A mash of spleen or liver from mice given 900 r immediately prior to the removal of these organs had a less marked effect on growth.

The growth of irradiated bacteria in intimate contact with growing, non-irradiated cells was observed in 2 experiments. A streptomycin-resistant variant of the *E. coli* strain was produced by serial passage of the strain through graduated streptomycin-containing broths. This variant was irradiated and then incubated together with a vigorously-growing unmodified culture. Colony counts were made as before, but with the incorporation of streptomycin in such concentration as to completely inhibit the non-irradiated streptomycin-sensitive strain, while permitting normal colony formation by the irradiated, streptomycin-resistant strain. In this way it was possible to follow the growth of the originally irradiated bacteria in cultures in which non-irradiated bacteria predominated.

The growth of irradiated and non-irradiated bacteria was also observed in the chorio-allantois of 10-day embryonated hens' eggs. Control observations were made on eggs that had been irradiated with 1000 r on the assumption that any substances with radiation-recovery potency would be destroyed in such eggs. No increase in the growth rates of irradiated bacteria could be demonstrated in either of these experiments.

Discussion. Although the lag phase of *E. coli* cultures given a single large exposure to X radiation is prolonged, the logarithmic growth rate is identical in irradiated and control cultures. The early inhibition of multiplication is thus completely reversible and therefore does not appear to represent the development of a stable mutant. It is clearly not the result of any persistent toxic changes in the media. The rapidity of resumption of normal growth varies directly with the temperature and inversely with the size of the X-ray exposure. This phenomenon is not limited to the strain of *E. coli* used in these experiments: we have also observed it in cultures of *Diplococcus pneumoniae* and strain B/R of *E. coli*‡(3). Because it occurs in cultures held at 0° and 37°C throughout, it appears to be unrelated to the recovery from the lethal effect of irradiation that has been described by Hollaender(4,5) as occurring in certain media only at intermediate temperatures.

It is well established that irradiation delays cellular division in many species(6,7). The effect of irradiation on the multiplication of bacteria has been reported in detail for cultures undergoing continuous irradiation during growth(8,9). Lea, Coulson, and Haines (9) have suggested that the failure of the viable counts of such cultures to increase may be due to a reversible delay of cellular division, rather than growth, so that long forms that subsequently divide are formed.

Although there is considerable evidence that the spleen and certain other organs elaborate a humoral substance or substances that will enhance recovery of irradiated mammals(10), no satisfactory means has been developed for assaying such materials in laboratory animals. This lack of success may, at least in part, be ascribed to the great dilution of injected materials in body fluids and to the relatively limited number of animals available. From these points of view the use of bacteria for assay is advantageous. Changes in the mortality rates of irradiated bacteria are readily demonstrated by altering the concentration of cysteine, oxygen, alcohols, pyruvate, and numerous other substances in the suspending medium *before* and *during* irradiation(5,11). Except for studies on the recovery of bacteria at intermediate temperature(5), the efficacy of any material added *after* irradiation has not been reported.

It must be emphasized that our studies have been concerned with a *sublethal* rather than a *lethal* effect of X rays on bacteria. Since only colony counts (viable counts) have been made, the growth curves describe only the survivors. The prolongation of the lag phase differs from the lethal effect of irradiation in that it is inherently reversible. It is not an all-or-none phenomenon. Fundamental differences are also suggested by the failure of pre-treatment with cysteine to affect the prolongation of the lag phase, while the influence of this compound on the lethal effects of X radiation is well known(11). It seems quite likely, therefore, that the mechanisms of these types of injury may be totally different.

Other studies have failed to show the influence of any substance, added after irradiation, on the *lethal* effects of X rays; in this study we have measured the effect of various substances added after irradiation on *recovery* from the lag phase. Although some positive effect on growth was achieved by the addition of spleen or liver mash, this effect was not limited to irradiated cultures. Furthermore, the effect was only partially destroyed by irradiation, and an effect of equal magnitude could be obtained through the use of a mash made from mouse kidney, which does not appear to enhance recovery from irradiation in mammals. For these reasons it seems unlikely, although not impossible, that the growth-promoting activity that has been observed represents the humoral factor that has been implicated in recovery of mammals from the damaging effects of ionizing radiations.

---

‡ Supplied to us by courtesy of G. E. Stapleton and A. Hollaender, Oak Ridge National Laboratory, Oak Ridge, Tenn.

Summary. 1. A prolongation of the lag phase in irradiated *E. coli* is described. The modifying effects of temperature during growth, storage of irradiated organisms, and the addition of mouse blood, inactivated rabbit serum, 0.5% cysteine solution, the filtrate of actively growing *E. coli* cultures, fresh spleen, liver and kidney mash, or growing non-irradiated *E. coli* cultures, have been studied. 2. Evidence that sublethal effects of X irradiation in bacteria differ from lethal effects is discussed.

---

1. Robinson, R. F., Phillips, M. D., and Nagelsen, M. G., A bibliography on the effects of X ray on bacteria, Battelle Memorial Inst., Aug. 20, 1951.

2. Stapleton, G. E., Hollaender, A., and Martin, F. L., *J. Cell. Comp. Physiol.*, 1952, v39, sup. 1.

3. Beutler, E., and Robson, M. J., unpublished data.

4. Stapleton, G. E., Billen, D., and Hollaender, A., *J. Cell. Comp. Physiol.*, 1953, v41, 345.

5. Hollaender, A., and Stapleton, G. E., *Physiol. Rev.*, 1953, v33, 77.

6. Giese, A. C., *Quart. Rev. Biol.*, 1947, v22, 253.

7. Lea, D. E., *Actions of Radiation on Living Cells*, Cambridge, England, University Press, 1946.

8. Rubin, B. A., *The relation of nucleic acid metabolism to the genetic effect of radiation in bacteria*, NSA, 1951, v5, 4953, AECU-1436.

9. Lea, D. E., Haines, R. B., and Coulson, C. A., *Proc. Roy. Soc.*, London, 1937, vB123, 1.

10. Jacobson, L. O., *Cancer Research*, 1952, v5, 315.

11. Hollaender, A., Stapleton, G. E., Burnett, W. T., Jr., *The Modification of X ray Sensitivity by Chemicals*, "Isotopes in Biochemistry, a Ciba Foundation Conference," Churchill Ltd., London, 1951, 96.

Received April 2, 1954. P.S.E.B.M., 1954, v85.

Ribonuclease Activity of the Mouse Pancreas. (20993)

M. RABINOVITCH.* (Introduced by I. Gersh.)

From the Departments of Anatomy and Medicine, University of Chicago, Chicago, Ill.

Although the activities of several enzymes have been studied in the pancreas after fasting(1-3) and after the administration of cholinergic drugs to normal animals(3-6), no data bearing on pancreatic ribonuclease could be found. Information on this enzyme is of particular interest because of the occurrence of large amounts of ribonucleic acid(7) and because the pancreas is a rich source of crystalline ribonuclease. The evidence presented in this paper indicates that ribonuclease activity of pancreatic homogenates is reduced by fasting and by the administration of pilocarpine.†

Materials and methods. Adult white mice fed Purina dog chow were used. Males were studied, with the exception of those in Exp. I, Table I. In the fasting experiments the mice received only water for the times indicated. For the pilocarpine experiments, the fasted or unfasted mice regardless of body weight were injected intravenously with 2 doses of 0.6 mg each of pilocarpine hydrochloride (total volume 0.6 ml) at 30 minute intervals. Controls were similarly injected with distilled water. Intense salivation, lacrimation and diarrheic stools followed the injections. The mice were killed by a blow on the head, and weighed. The pancreas was removed, and weighed on a torsion balance. 0.1% homogenates were prepared with chilled distilled water, and the homogenates stored at –10°C until the determinations of enzyme activity were performed, within the next 18 hours. Control and pilocarpine-treated mice from each experiment were injected and killed alternately, and determinations of enzyme activity were performed simultaneously. Comparisons are to be made only within the individual experi-

---

* Fellow of the Rockefeller Foundation. Permanent address: Dept. of Histology and Embryology, Faculty of Medicine, University of São Paulo, Brazil.

† After completion of this paper a preliminary communication appeared(8) which claims that a reduction of the ribonuclease activity of the mouse takes place after the injection of carbamyl choline.

TABLE I. Influence of Fasting on the Ribonuclease Activity of the Pancreas of the Mouse.

Exp.	Treatment	No. of animals	Avg wt in g	Ribonuclease activity*	Stand. dev.
I †	a) Control	13	26.8	.264	.038
	b) 12 hr fast	7	29.1	.256	.045
	c) 24 " "	6	26.8	.194	.015
II‡	a) Control	10	28.8	.208	.042
	b) 30 hr fast	10	22.7	.165	.040

* Increase in opt. dens. at 260 mμ, produced by 13.3 μg fresh tissue.

† F found 8.24, $P < 1\%$; t tests, groups a-b, $P > 0.6$; a-c, $P < 0.001$; b-c, $P < 0.01$.

‡ $t = 2.35$, $P < 0.05 > 0.02$.

ments. *Ribonuclease activity* determinations were made by reading at 260 mμ(9) in a Beckman model DU spectrophotometer the absorption due to soluble nucleotides liberated from yeast nucleic acid by enzymatic activity of the homogenate. The composition of the substrate was the following: 0.5 ml of 0.05 M phosphate buffer pH 7.3; 0.3 ml of a 0.35% solution of commercial yeast ribonucleic acid, purified according to Kunitz(10) dissolved in 0.1 M sodium acetate and dialysed against 0.1 M sodium acetate at 4°C for at least 48 hours before use. To this substrate was added 0.2 ml of homogenate diluted 1:15, and the mixture was incubated for 20 minutes at 37°. Enzyme activity was interrupted, and undigested ribonucleic acid precipitated by the addition of 2 ml of 1 N HCl in 80% alcohol(11). After agitation and standing for one hour at 4°C, the tubes were centrifuged for 15 minutes in a refrigerated centrifuge, and aliquots of the supernatants taken for spectrophotometry. Blanks were run by adding the homogenates to the substrate after addition of the acid alcohol. Results are expressed in increase of the optical densities over those of the blanks, when an equivalent of 13.3 micrograms of fresh tissue was used for the determinations. In experiments with crystalline ribonuclease it was found that an optical density increase of 0.200 over the blanks was obtained with 0.005 μg of the enzyme. Previous results(3,12), did not disclose significant wet weight/dry weight ratio modifications in the mouse pancreas after the administration of pilocarpine.

Under these conditions of enzyme determination, it was found that there was a direct proportionality between the amount of pancreatic tissue used and enzyme activity. Activity of the homogenates was not modified by their storage at 4°C for 48 hours. Magnesium sulfate (final concentration 0.06 M) led to 50% inhibition of the activity of the homogenates, which agrees with previous data obtained with the crystalline enzyme(13). No modification in the activity of the homogenates was found when they were prepared with either 0.1% "Versene" solution (pH around 6), 0.1% saponin (pH around 7.0) or 0.15 N H_2SO_4.

Results. Table I shows that 24 and 30 hours, but not 12 hours, of fasting decreased the ribonuclease activity of the pancreas. Table II shows that one hour after the administration of pilocarpine the average ribonuclease activity of the pancreas is decreased significantly. Twelve hours after the administration of pilocarpine (exp. III), the ribonuclease activity of the pancreas has recovered to normal values of control mice.

Discussion. The decrease in ribonuclease activity of the pancreas in fasting mice agrees with the findings of others on protease(1,2), amylase(2,3) and lipase(2). The biochemical assays also agree with the histological descriptions of Okuneff(14), who reported reduction in the number of zymogen granules in the rabbit pancreas after prolonged fasting. The early return to normal levels after only 12 hours following the injection of pilocarpine also corresponds with the time when the secretory granules reach their normal density in the apical pole of the acinar cells. It is interesting also that Marshall(15) was able to localize ribonuclease in the apical region by a specific cytochemical method. For these reasons, it is possible to suggest that at least part of the ribonuclease in the pancreas is excreted in the pancreatic juice. The possi-

TABLE II. Influence of Pilocarpine Administration on the Ribonuclease Activity of the Pancreas of the Mouse. 4 experiments.

Time (hr) after 1st inj.	Treatment	No. of animals	Avg wt in g	Ribonuclease activity*	Stand. dev.	Probabilities (t test)
1	U.c.†	10	17.6	.183	.043	$<.01$
	P.	9	16.8	.120	.025	
1	U.c.	13	23.4	.192	.038	$<.001$
	P.	12	23.2	.128	.030	
12	U.c.	10	27.1	.169	.039	$>.7$
	P.	9	26.0	.175	.057	
1	6 hr F.c.	11	24.6	.214	.077	$\overline{>}.05$
	P.	11	25.2	.156	.053	

* Increase in optical density at 260 mμ produced by 13.3 μg fresh tissue.
† U.c. = unfasted controls; P. = pilocarpine; F.c. = fasting controls.

bility of vascular discharge cannot be excluded(16).

Summary. Twenty-four and 30 hours fasting reduces ribonuclease activity of the pancreas of mice. One hour after the administration of pilocarpine a significant drop of enzymic activity takes place, with recovery in less than 12 hours.

The author is indebted to Professors I. Gersh and E. S. G. Barron for helpful suggestions.

---

1. Heidenhain, R., *Arch. ges. Physiol.*, 1875, v10, 557.

2. Bocchioti, S., *Bol. Soc. Ital. Biol. Sper.*, 1948, v24, 952.

3. Daly, M. M., and Mirsky, A. E., *J. Gen. Physiol.*, 1952, v36, 243.

4. van Weel, P. B., *Z. vergl. Physiol.*, 1939, v27, 311.

5. van Weel, P. B., and Engel, C., *ibid.*, 1938, v26, 67.

6. Hokin, L. E., *Biochem. J.*, 1951, v48, 320.

7. Davidson, J. N., *The Biochemistry of the Nucleic Acids*, Methuen, London, 1950.

8. Dickman, S. R., and Kropf, R., *Fed. Proc.*, 1954, v13, 199.

9. Schneider, W. C., and Hogeboom, G. H., *J. Biol. Chem.*, 1952, v198, 155.

10. Kunitz, M., *J. Gen. Physiol.*, 1940, v24, 15.

11. Roth, J. S., and Milstein, S. W., *J. Biol. Chem.*, 1952, v196, 489.

12. Rabinovitch, M., Valeri, V., Rothschild, H. A., Camara, S., Sesso, A., and Junqueira, L. C. U., *ibid.*, 1952, v198, 815.

13. Lamanna, C., and Mallette, M. F., *Arch. Biochem.*, 1949, v24, 451.

14. Okuneff, N., *Arch. mikr. Anat.*, 1923, v97, 187.

15. Marshall, Jr., J. M., *Exp. Cell. Res.*, 1954, v6, 240.

16. Popper, H. L., and Necheles, H., *Gastroenterology*, 1943, v1, 490.

Received April 6, 1954. P.S.E.B.M., 1954, v85.

AUTHORS' INDEX

VOLUME 85

(*The numerals indicate the page*)

Abramson, H. A. 255.
Adams, C. H. 18.
Adler, M. 214.
Adler, T. K. 77.
Albaum, H. G. 231.
Aldrich, R. A. 573.
Anderson, H. H. 77.
Arnold, J. S. 113, 658.
Aronson, S. M. 214.
Arteta, J. L. 162.
Austoni, M. E. 48.

Baeder, D. H. 160, 444.
Baker, B. L. 635.
Bakst, H. 329.
Balsam, T. 21.
Barden, R. 463.
Barraclough, C. A. 673.
Bartlett, R. G., Jr. 81.
Baumann, C. A. 561.
Baxter, J. A. 648.
Beard, D. 406.
Beard, J. W. 406.
Bearn, A. G. 44.
Beary, D. F. 432.
Beckfield, W. J. 444.
Berg, W. E. 606.
Berger, M. 89.
Bernheim, F. 542.
Berry, L. J. 209.
Bessman, A. N. 66.
Bessman, S. P. 66.
Beutler, E. 682.
Bew, E., Jr., 96.
Biehl, J. P. 389.
Bjorklund, B. 438.
Black, M. M. 11.
Bloch, E. 106.
Block, W. D. 220.
Board, J. A. 37.
Bocobo, F. C. 220.
Bodine, J. H. 156, 322.
Bohr, V. C. 54.
Boltralik, J. J. 157.
Bongiovanni, A. M. 428.
Bothwell, J. W. 544.
Bovell, C. 495.
Boyle, E. 468.
Bradwick, K. 333.
Braun, W. 154.
Briggs, G. M. 451.
Broderick, M. K. 128.
Brown, G. C. 83, 104.
Browne, K. M. 474.
Butler, L. C. 139, 441.
Byers, S. O. 530.

Cabasso, V. J. 167, 239.
Camien, M. N. 177.
Campbell, R. M. 398.
Cantarow, A. 422.
Carbone, J. V. 101.
Carhart, E. 248.
Carlson, Loren D. 156.
Carlson, L. D. 303.
Carlson, W. W. 211.
Case, J. D. 573.
Cavazos, L. F. 511.
Chamberlain, R. W. 667.
Chen, G. 245.
Chernin, E. 68.
Childs, R. 96.
Chinn, H. I. 472.
Christensen, H. N. 388.
Chrystowski, G. A. 190.
Churg, J. 615.
Clayton, G. W., Jr. 428.
Cochran, K. W. 104.
Cohen, C. 375.
Cole, J. W. 631.
Comar, C. L. 292.
Constant, M. A. 678.
Cook, R., Jr. 120.
Cooley, S. L. 409.
Coover, M. O. 317.
Cornatzer, W. E. 642.
Cosgrove, R. 218.
Co Tui 398.
Couch, J. R. 645.
Cowperthwaite, J. 117.
Cox, H. R. 167, 239.
Crosby, W. H. 101.
Curtis, A. C. 220.

D'Angelo, S. A. 633.
Dasler, W. 485.
Daugherty, J. W. 288.
Day, P. L. 280, 460.
Day, R. L. 261.
Denison, M. E. 433.
DesAutels, E. J. 643.
Desmedt, J. E. 491.
Dhyse, F. G. 515.
Dinning, J. S. 280.
Doan, C. A. 295.
Dorfman, R. I. 106.
Dorman, D. C. 454.
Douglass, C. D. 460.
Dowe, T. W. 18.
Downey, G. J. 643.
Duncan, G. M. 37.
Dunn, M. S. 177.
Dwyer, P. 75.

Earl, A. E. 323.
Eckert, E. A. 406.
Edgren, R. A. 229.
Eischeid, A. M. 339.
Elam, J. F. 645
Elliott, H. W. 77.
Ellis, J. 507.
Elvehjem, C. A. 42.
Emerson, K., Jr. 264.
Enright, J. B. 466.
Eskelson, C. D. 637.

Fand, I. 398.
Farah, A. 248.
Fasoli, A. 609.
Favour, C. B. 237.

Fazekas, J. F. 66.
Feigenbaum, L. 530.
Fell, C. 199.
Feng, Y. S. L. 341.
Fenn, W. O. 75.
Fink, M. A. 336.
Finland, M. 25, 40, 61, 128.
Firschein, H. E. 637.
Firth, J. 307.
Fisher, M. W. 538.
Fleischmann, W. 655.
Flesch, P. 483.
Floyd, E. P. 206.
Flux, D. S. 16.
Foa, P. P. 609.
Forbes, J. C. 37.
Foster, M. 120.
Fowler, J. 645.
Francis, T., Jr. 83, 104.
Francis, Y. F. 457.
Freeman, S. 125.
Friedman, B. K. 282.
Friedman, M. 530.
Frisch, A. W. 573.
Fruhman, G. J. 93.
Fuller, H. S. 151.
Furcolow, M. L. 72.
Furth, F. W. 101.

Gaffey, C. T. 144.
Gagnon, J. 576.
Gal, E. M. 252.
Gamble, J. L., Jr. 172.
Gandia, H. 234.
Georgi, C. E. 137.
Gerard, R. W. 174.
Gerschman, R. 75.
Gerstner, R. 93.
Gettner, H. H. 255.
Gibbons, C. A. 51.
Gilbert, D. L. 75.
Ginsberg, H. S. 192.
Gittler, R. 329.
Glassman, M. D. 609.
Glick, C. 96.
Glocklin, V. C. 663.
Goldenberg, H. 275.
Goldfarb, A. R. 255.
Goldie, H. 578.
Goldman, R. 446.
Goldner, M. G. 347.
Goltz, H. L. 367.
Gomori, G. 570.
Goodman, J. W. 500.
Gordon, A. S. 93.
Gorzynski, E. A. 503.
Gould, D. J. 558.
Gourzis, J. T. 463.
Green, I. 406.
Greenberg, D. M. 252.
Greif, R. L. 674.
Grobstein, C. 477.
Grollman, A. 582.
Grossman, M. I. 356.
Grunt, J. A. 540.
Gump, H. P. 412.

Hackel, B. 593.
Haley, T. J. 258.
Hallinger, L. N. 624.
Hanan, R. 373.
Harrell, E. R. 220.
Haslam, R. 401.
Havel, R. J. 468.
Hechter, O. 583.
Heglin, J. 258.
Helmendach, R. H. 54, 81.
Hennessen, W. A. 359.
Herman, C. M. 272.
Heymann, W. 593.
Hilleman, M. R. 183.
Hoene, R. 56.
Hoffman, J. G. 367.
Holley, H. L. 665.
Hsia, D. Y. 86.
Huseby, R. A. 422.

Ibach, M. J. 72.
Ingle, D. J. 432, 635.
Inman, W. I. 81.
Ivanovic, N. 199, 496.

Jacobs, R. L. 645.
Jacobson, L. O. 682.
James, M. F. 307.
Jandl, J. H. 166.
Jauregui, R. H. 347.
Jee, W. S. S. 658.
Jefferson, N. C. 67.
Jensen, H. 637.
Johnson, B. C. 307.
Johnson, J. F. 496.
Johnson, S. A. 199.
Jones, A. M. 578.
Jones, F. 1.
Jordan, W. S., Jr. 648.
Joseph, L. 188.

Kagan, B. M. 457.
Kagawa, C. M. 393.
Kaiser, E. 457.
Kandel, A. 83.
Kaplan, E. H. 142.
Kass, E. H. 583.
Keating, R. P. 420.
Kelley, B. 460.
Kelly, D. R. 123.
Kelsh, J. 154.
Kensler, C. J. 364.
Kent, J. F. 635.
Kilham, L. 272.
Kind, L. S. 371.
Klement, A. W., Jr. 172.
Knowlton, A. I. 627.
Kochakian, C. D. 339.
Koda, F. 248.
Kraintz, F. W. 416.
Krause, R. F. 317.
Krech, U. 344.
Kuhlman, R. E. 458.
Kunkel, H. G. 44.
Kuyper, A. C. 4.

Ladd, M. 576.
Lamden, M. P. 190.
Langemann, H. 364.
Lardy, H. A. 89.
Larsh, H. W. 72.
Leathem, J. H. 673.
Lee, J. M. 430.

Lehr, D. 615.
Leoschke, W. L. 42.
Lewis, Y. S. 306.
Li, C. H. 635.
Lindgren, F. T. 352.
Little, R. C. 639.
Lloyd, C. W. 333.
Loeb, E. N. 627.
Loewy, E. 333.
Loring, W. E. 350.
Lotz, W. E. 292.
Loughry, C. W. 631.
Love, B. D., Jr. 25.
Ludwig, A. W. 424.
Luisada-Opper, A. V. 482.

Macchi, I. A. 583.
Macri, F. J. 603.
Maddock, C. L. 598.
Magid, E. B. 609.
Marvin, J. F. 379.
Matsushima, J. 18.
McCulloh, E. 258.
McGrath, J. 96.
McQueen, J. D. 474.
McShan, W. H. 393.
Meisel, E. 314.
Meites, J. 341.
Melampy, R. M. 511.
Menaker, W. 149.
Mendlowitz, M. 111.
Meschan, I. 460.
Meyer, R. K. 393.
Miesse, M. L. 558.
Mikuta, E. T. 29.
Militzer, W. 137.
Miller, W. L., Jr. 561.
Milora, R. 615
Mirand, E. A. 367.
Mistry, S. P. 307.
Mitchell, R. B. 209.
Mitchell, W. G. 206.
Montgomery, V. 596.
Moon, V. H. 7, 600.
Moore, D. H. 255.
Morgan, A. F. 139, 441.
Morgan, C. C. 211.
Morgan, M. C. 420.
Morita, T. N. 188.
Moses, C. 590.
Mou, T. W. 25, 61, 583.
Muirhead, E. E. 1.
Mullertz, S. 326.
Mullins, L. J. 144.

Napoli, M. D. 400.
Narahara, H. T. 51.
Nathan, H. A. 117.
Neter, E. 503.
Neumann, G. 624.
Neve, R. A. 573.
Nichols, A. V. 352.
Nimaroff, M. 655.
Norton, T. W. 239.
Nye, S. W. 75.

O'Dell, T. B. 400.
Olitsky, P. K. 430.
Olwin, J. H. 496.
Opdyke, D. F. 414.
Ortiz, L. O. 451.
Ott, W. H. 414.
Oyama, J. 373.

Pace, N. 553.
Page, M. 86.
Parker, G. 477.
Paschkis, K. E. 422.
Phillips, P. H. 678.
Pierog, S. 333.
Pincus, G. 106.
Pitts, F. W. 404.
Platt, D. 665.
Polson, A. 613.
Ponsford, J. 454.
Popper, H. L. 67.
Porter, J. C. 511.
Portman, R. 245.
Powell, L. T. 317.
Prevedel, A. E. 596.
Price, W. C. 157.
Prusoff, W. H. 564, 567.
Purcell, E. M. 25, 61, 128.
Purmalis, A. 432.

Rabinovitch, M. 685.
Rapaport, H. G. 255.
Rapport, M. M. 282.
Rather, L. J. 285.
Reid, M. E. 547.
Reinhard, M. C. 367.
Reisner, E. H., Jr. 420.
Rennels, E. G. 309.
Rennick, B. R. 234.
Riggs, H. E. 96.
Riggs, T. R. 388.
Rinzler, S. H. 329.
Robinson, A. R. 4.
Robson, M. J. 682.
Rogers, E. B. 172.
Root, M. A. 507.
Rosen, W. G. 385.
Rosenthall, L. 379.
Ross, D. E. 578.
Roth, K. L. 533.
Rothbard, S. 133.
Rothburd, M. 398.
Rothlauf, M. V. 336.
Rubin, L. 352.
Ruskin, A. 309.
Rust, J. H. 258.

Sabin, A. B. 359.
Sadler, W. W. 466.
Saslaw, S. 223, 295.
Satanove, A. 483.
Savitsky, J. P. 587.
Schaefer, J. 223.
Schaefer, R. D. 639.
Scheid, H. E. 382.
Schiff, L. 401.
Schneider, H. A. 430.
Schneider, J. A. 323.
Schneider, M. D. 32.
Schnitzer, R. J. 123.
Schwarz, H. P. 96.
Schweigert, B. S. 382.
Scott, R., Jr. 101.
Seegers, W. H. 199, 496.
Seifter, J. 160, 444.

Selby, P. N., Jr. 388.
Seronde, J., Jr. 521.
Shaffer, J. G. 21.
Sherry, S. 587.
Shils, M. E. 298.
Shwartzman, G. 214.
Sikes, R. K. 667.
Silber, R. 624.
Silberberg, M. 448.
Silberberg, R. 448.
Simmons, M. R. 280, 460.
Sklarofsky, B. 255.
Smith, D. E. 306.
Smith, F. 412.
Smith, L. C. 643.
Smith, O. W. 264.
Sobel, A. E. 275.
Sobotka, H. 482.
Sonnenschein, R. R. 463.
Sostheim, R. 333.
Speer, F. D. 11.
Spivey, M. R. 451.
Stanley, N. F. 454.
Staub, A. 507.
Stavitsky, A. B. 593.
Stebbins, M. R. 167, 239.
Stein, I. 329.
Stevens, C. E. 633.
Stevenson, T. D. 295.
Stewart, W. B. 298.
Stoerk, H. C. 627.
Stone, F. 96.
Streicher, E. 174.
Strub, I. H. 356.
Stulberg, C. S. 4.
Swan, H. 596.

Takemoto, K. 670.
Talmage, R. V. 292, 416.
Talman, E. L. 573.
Taylor, S. P., Jr. 226.
Teodoru, C. V. 214.
Tershakovec, G. A. 7.
Tershakovec, M. A. 600.
Tewell, H. E., Jr. 125.
Thomas, R. S. 553.
Thomson, J. F. 29.
Tillotson, I. G. 192.
Tittler, I. A. 495.
Tolbert, B. M. 77.
Tomizawa, H. H. 51.
Travell, J. 329.
Trum, B. F. 258.
Turner, C. W. 524.

Ullberg, S. 550.

Vaughan, B. E. 553.
Vilter, R. W. 389.

Wachstein, M. 314.
Walker, A. E. 474.
Walker, E. L. 553.
Walker, M. 578.
Wang, H. 382.
Wang, S. C. 472.
Ward, J. 401.
Warren, J. 359.
Watson, R. F. 133.
Weimar, V. 488.
Weinstein, J. 329.
Weller, T. H. 68.
Werner, J. H. 183.
West, W. L. 322.
White, R. 137.
Whitehorn, W. V. 268.
Wick, A. N. 188.
Wilcox, C. 128.
Williams, J. N., Jr. 544.
Williams, R. H. 51.
Williams, W. F. 524.
Wilson, J. G. 319.
Wilson, L. 652.
Wood, J. L. 409.
Wright, S. S. 25, 40, 61, 128.

Yielding, K. L. 665.
Young, J. M., Jr. 280.
Youngner, J. S. 202, 527

Zahl, P. A. 231.
Zarrow, M. X. 433.
Zinder, N. D. 430.
Zucker, L. M. 517.
Zucker, M. B. 282.
Zucker, T. F. 517.
Zweifach, B. W. 11.

SUBJECT INDEX

VOLUME 85

(*The numerals indicate the page*)

Acid, adenylic, nucleotide level. 231.
  amino, and carboxylic, fibrinolysis. 326.
    and growth. 409.
    by *Hymenolepis*. 288.
  ascorbic, oxalate excretion. 190
  hyaluronic, spreading action. 160.
    streptococcal. 444.
  iodoorotic, microbiological activity. 567.
  keto and growth. 409.
Adrenalectomy, tuberculin reaction. 237.
Adreno-cortical hyperplasia, hormones. 428.
Alkaloids, *Rauwolfia serpentina* effect on heart. 463.
Ammonium compounds, bis-fluorenyl-bis-quaternary. 603.
Amyloidosis, Freund-type adjuvant. 133.
Anaphylaxis, sensitivity, histamine. 336.
Antibiotic, chloramphenicol, peripheral or bone marrow picture. 295.
  cross resistance, *E. coli*. 128.
  chlortetracycline, antipyretic action. 192.
    cattle scours. 18.
    on growth. 379.
    oxytetracycline, tetracycline. 25, 40, 61.
  effect on poliomyelitis. 104.
  oxytetracycline, chlortetracycline, tetracycline. 25, 40, 61.
  penicillin, growth promoting. 645.
    S^{35}, radioautography. 550.
  streptomycin, plant growth inhibition. 385.
  tetracyclines, absorption, excretion. 61
    antibacterial action. 25.
    cross resistance. 40.
Antibody, bovine encephalomyelitis. 466.
  gel precipitate. 438.
  isohemagglutinin production. 375.
  x-irradiation. 412.
Antigen-antibody reaction. 593.
Antigen, *Shigella dysenteriae*. 503.
  size, cortisone. 373.
Arginine, microbiological assay. 177.
Arthritis, agglutination test. 4.
Atabrine, leucopenia from x-irradiation. 460.

Bacterium, *Brucella,* blood cultures. 154.
  corynebacterium, pantothenic acid deficiency. 521.
  E. coli, biotinless mutant, growth inhibition. 89.
    lag phase. 682.
  Salmonella typhimurium, malonate. 209.
  Streptococcus faecalis, thymidine. 564.
  Streptococcus pyogenes. 444.
BCG, isohemagglutinin production. 375.
Bilirubin, brain respiration. 261.
Bilirubinemia, virus isolated. 272.
Blood, anemia, muscular stiffness disease. 488.
  antidiuretic material. 333.
  antithrombin activity. 199, 496.
  cells, cortisone, thermal stress effects. 7.
    phagocytosis *Endamoeba histolytica*. 21.
  clot retraction time. 587.
  clotting factors. 533.
  eosinophils, cortisone acetate. 433.
    pyridoxine deficiency. 139.
  erythrocyte-modifying study. 503.
  erythrocytes and pyridoxal. 388.
  erythromyeloblastic leukosis. 406.
  erythropoiesis, growth hormone effect. 93.
  fat emulsions. 356.
  hemolytic reaction, magnesium. 648.
  leucocyte changes, pyridoxine deficiency. 441.
    vitamin deficiency. 280.
  leucocytosis, muscular stiffness disease. 488.
  leucopenia, x-irradiation. 460.
  lipemia clearing factor. 468.
  nucleotide, tumor bearing mice. 231.
  plasma alloxan, glucose. 482.
    lipoprotein changes. 553.
  platelets, adhesiveness. 587.
    I^{131}. 420.
    irradiation. 258.
    serotonin. 282.
    suspension, lipemic plasma. 631.
  pressure, sodium azide effect. 11.
  serum lipoprotein, depancreatized dogs. 609.
  sperm agglutinins. 652.
  sugar, ergotamine tartrate. 218.
  thyroid hormone level. 637.
  volume determination. 350.
Brain, hypothalamus, hypertension. 474.
  respiration, bilirubin. 261.
5-Bromouracil, thymidine, *Streptococcus faecalis*. 564.

Calcification, cation linked inhibition. 275.
Calorimeter, digital. 111.
Cartilage, embryonic spinal cord. 477.
Casein, iodinated histological studies. 382.
Cholesterol, avian atherosclerosis. 414.
  reticulo-endothelial system. 530.
Chromatography, oxytocin, vasopressin. 226.
Chromogen, fermentable, renal clearance. 576.
Ciliate, sulfonamides on thiamine requirements. 495.
Clostridia, dietary, growth promoting. 645.
Cold sensitivity, stimulus. 303.
Coproporphyrin, urinary excretion. 86.
Cottonseed oil, avian atherosclerosis. 414.
Creatinine, renal failure. 446.

Dextran, hemostatic defect. 101.
Diabetogenic effect of glucagon. 432.
Dicumarol, antithrombin activity. 496.
Diet, atherogenic. 414.
  corn, development of fatty liver. 298.
  erythrocyte fragility, X-radiation. 678.
  high fat and protein, B_{12} deficiency. 451.
    in gelatine and casein, odoratism. 485.
  protein and pancreatic damage. 314.
Dimethylethanolamine, toxicity. 642.
2,4 Dinitrophenol, reagents. 322.
Diuretics, mercurial, succinic dehydrogenase. 309.
Drains, Penrose and Teflon. 404.
Dyes, azo blue cause congenital malformations. 319.

Embryo, mesoderm, spinal cord. 477.
Emesis after irradiation. 472.
Enzyme, aspartic-glutamic transaminase. 339.
  ATPase measurements on plasma. 406.
  choline metabolism and liver. 364.
  effect of quinones. 542.
  fibrinolysin and proteins. 142.
  hexose-6-phosphatase, reproductive system. 458.
  hyaluronidase, blood clotting. 533.
  lysine deficiency. 544.
  peptidases, *Mytilus edulis*. 606.
  protyrosinase, U. V. spectrum. 156.
  ribonuclease, pancreas. 685.
  succinic dehydrogenase, kidney. 309.
    testis. 674.
  tyrosinase, histochemistry. 120.
    U. V. spectrum. 156.
Epinephrine, thyroid hormone. 637.
Ergonovine, cardiac pain. 329.
Ergotamine tartrate, blood sugar. 218.
Ethanol oxidation, antabuse. 252.
Ethyl chloride spray, cardiac pain. 329.
Ethylene diamine tetraacetate, vanadium poisoning. 206.
Excitability, hyper, castrated male. 540.
Exophthalmos, vitamin C deficiency. 424.

Fibrinolysis, effect of carboxylic and amino acids. 326.
Fluoroacetate sodium, and poliomyelitis. 83.
Fungi, anti activity of nitrostyrenes. 220.

Gel precipitates, color reactions. 438.
Glucagon, depancreatized rats. 432.
  effect on insulin hypoglycemia. 507.
Glucose, effect of histamine, epinephrine and anti-histamine. 347.
    on plasma alloxan. 482.
Glycol mesylates, sarcoma 180. 211.
Growth hormone, erythropoiesis. 93.
  keto and amino acid relationship. 409.

Heart, aortic disease. 285.
  cardiac arrest, hypercapnia, vagal asystole. 54.
    pain, ergonovine. 329.
  cardiovascular necrosis. 615.
  muscle, tetanus. 268.
  Rauwolfia serpentina effect. 463.
  ventricular contractions. 639.
    fibrillation, prostigmine. 596.
Heparin, fat emulsions. 356.
  interaction, lipoprotein. 352.
  lipemia clearing factor. 468.
  lipid metabolism. 468.
Heredity, encephalomyelitis. 430.
Histamine, anaphylactic shock. 336.
  cortisone antagonism. 371.
Histoplasma capsulatum in air. 72.
Histoplasmosis, resistance. 223.
Hormone, ACTH, melanophorotropic potency. 229.
    plasma proteins. 643.
    treatment. 583.
  adrenal, salicylates effect. 37.
  androgen, hyperexcitability. 540.
  assay, *Hyla cinerea*. 229.
  chromatography. 226.
  corticosteroids, ACTH. 583.
    17-ketosteroids, excretion. 125.
  corticotropin, leucocytes. 635.
  cortisone acetate. 433.
    and antigen size. 373.
    brown fat. 214.
    capillary permeability. 600.
    effect on enzymes. 339.
    -histamine antagonism. 371.
    hydrocortisone in microbial growth. 166.
    in mammary cancer. 264.
    leucocytes. 635.
    liver feeding. 627.
    protein catabolic action. 341.
    scorbutus. 598.
    serous exudation. 578.
    serum. 582.
    thermal stress effects. 7.
  follicle-stimulating in pituitary. 393.
  glucocorticoids in tumors. 422.
  hydrocortisone, metabolism. 51.
  17-ketosteroids, adrenal perfusates. 106.
  lactogenic—I^{131} & mammary glands. 524.
  11-oxygenated neutral 17-ketosteroids. 428.
  parathyroid, radiocalcium and radiophosphorus. 292.
  testosterone, lipid distribution. 511.
    propionate, infertility. 673.
      sex organs. 655.
  thyroid, epinephrine effect. 637.
  urinary estrogens in mammary cancer. 264.
  17-vinyltestosterone, mammary growth. 16.
Hymenolepis, synthesis of amino nitrogen. 288.
Hypertension, alpha-tocopherol. 615.
  hypothalamic lesions. 474.
  renal, silica. 590.
Hypophysectomy, antidiuretic. 333.

Infection, corynebacterium and pantothenic acid deficiency. 521.
  pantothenic acid deficiency. 517.
Inflammations, hyperergic, protection. 162.
Injury, thermal, resistance. 56.
Insulin, hypoglycemia, glucagon. 507.
Intestine, peristaltic reflexes. 245.
Inulin, renal clearance. 576.
Iodide, 1,1-dimethyl-4-phenylpiperazinium. 245.
Iodouridine, microbiological activity. 567.
Ion exchange, Ca^{45}, skeletal. 658.
Isoniazid in peripheral neuritis. 389.
  revival of tubercle bacilli. 538.
Isotope, C^{14} carotene, extrahepato-intestinal tissue. 317.
  Ca^{45}, skeletal. 658.
  Cu^{64}, in serum. 44.
    in Wilson's disease. 44.
  Fe^{59}, in erythroid cells, autoradiography. 48.
  I^{131} and blood platelets. 420.
    localization of hormone. 524.
  P^{32} counting. 172.
    thyroid, biological decay. 633.
      deficiency. 448.
    brain phospholipids. 174.
    on growth. 379.
  S^{35}-labelled penicillin. 550.

Joint disease, thyroid, I^{131}. 448.

Kidney, chromogen, inulin. 576.

  circulation, smooth muscle valve. 234.
  creatinine. 446.
  mercurial diuretics. 309.
  operation, aortic disease. 285.
  slices, P-aminohippurate. 248.
  trypsin-dispersed. 202.
    poliomyelitis. 527.
  vitamin A metabolism. 457.

Lathyrism, diets high in gelatin and casein. 485.
Leptospires, antigens. 32.
Lipid, skin sterols, cholesterol. 561.
Liver, abscess, chlortetracycline effect. 18.
  choline metabolism. 364.
  disease, NH_3 and coma. 66.
  fatty, lipotropic agents. 298.
  feeding, counteract cortisone. 627.
  glucose turnover. 347.
  glycogen, infrared spectroscopy. 96.
  hepatic cirrhosis. 401.
  lysine deficiency, enzyme activity. 544.
  silica injection. 590.

Magnesium ions, cold hemoglobinuria. 648.
  protein synthesis. 149.
Malonate, effect on bacteremia. 209.
Manganese, plant growth. 385.
Mannitol, oxidation. 188.
Mast cell, total body irradiation. 306.
Mephenesin, muscle excitability. 500.
O-methoxyphenylglycerol ether on spinal reflex. 323.
Morphine-N-methyl-C^{14}, excretion. 77.
Muscle, excitability, Mephenesin. 500.
  valve, smooth,pharmacology. 234.

Naphthylamines, acyl, enzymatic hydrolysis. 570.
Nephrectomy, Na and K. 1.
Nerve, activity of deplanted nerve centers. 491.
  depolarization. 144.
Neuritis, B_6 and isoniazid. 389.
Nitrostyrenes, antifungal activity. 220.

Oxalate excretion, ascorbic acid. 190.
Oxygen poisoning, β-mercaptoethylamine. 75.

Pancreas, damage, protein free diet. 314.
  extract and diabetes. 432.
  ribonuclease activity. 685.
  serum lipoprotein, depancreatized dogs. 609.
Parathyroidectomy, phosphate excretion. 416.
Phosphate excretion, parathyroidectomy. 416.
Pituitary source of follicle-stimulating hormone. 393.
Polysaccharide, erythrocyte modifying capacity. 503.
Potassium concentration in joint fluid. 665.
  Na, nephrectomy. 1.
Propylene glycol, methemoglobin, p-chloroacetanilide. 663.
Prostigmine, ventricular fibrillation. 596.
Protein, catabolic action of cortisone. 341.
  lipo-, heparin factor. 352.
  synthesis, Mg and protein intake. 149.
Purines, pyrimidines, growth inhibition. 89.
Pyridoxal and erythrocytes. 388.
Pyrimidines, purines, growth inhibition. 89.

Quinone, effect on adaptive enzymes formation. 542.

Radiation, gamma ray. 258.
  strain differences. 367.
Radioautography, liquid emulsion. 113.
Ragweed extract, preparation. 255.
Reproductive system, composition of rat seminal vesicles. 511.
  hexose-6-phosphatase. 458.
Respiratory illness. 183.
Restraint, temperature regulation. 81.
Reticulo-endothelial system, cholesterol. 530.
Rickettsiae, spotted fever group. 558.
  typhus, serial passage, lice. 151.

Salicylates, adrenal response. 37.
Sedormid, effect on chick embryo. 573.
Semen, sperm agglutinins. 652.
Serum complement, antigen-antibody reaction. 593.
  exudation, peritoneal cavity. 578.
  levels, cortisone. 582.
Skin, cellular and keratinous components. 483.
Sodium concentration in joint fluid. 665.
  K, nephrectomy. 1.
Sulfonamides, *Tetrahymena geleii.* 495.
Synovial fluid, Na, K. 665.

Testis, enzyme distribution. 674.
Tetanus, cardiac muscle. 268.
Tetraethylthiuramdisulfide. 252.
Thymidine, 5-bromouracil. 564.
Thymus changes, pyridoxine deficiency. 441.
Thyroid, deficiency, I^{131}. 448.
  iodinated casein and Vit. B_{12}. 382.
Tissue culture, infectious canine hepatitis. 239.
  on skin-blood relationship. 398.
  poliomyelitis. 359.
  poliomyelitis virus assay. 527.
  trypsin-dispersed monkey kidney cells. 202.
Toxoplasma gonddii, propagation. 68.
Trace minerals, erythrocyte fragility, X-radiation. 678.
Trichomonas vaginalis, interference. 123.
Trypanosomid flagellate, antimalarial testing. 117.
Tubercle bacilli, isoniazid. 538.
Tuberculin, adrenalectomy effect. 237.
Tuberculosis, mycobacterium. 624.
  pulmonary, ACTH. 643.
Tumor, adrenocortical carcinomas. 422.
  cell, proliferation, peritoneal cavity. 578.
  mammary cancer, estrogens. 264.
  sarcoma 180, glycol mesylates. 211.

Urinary calculi, prevention, minks. 42.

Vanadium, toxicity, antidotes. 206.
Vein, portal, occlusion. 67.
Veratrum, emetic and hypotensive action. 400.
Virus, bacteriophage, thermophilic. 137.
  bilirubinemia and jaundice. 272.
  bovine encephalomyelitis antibodies. 466.
  eastern equine encephalitis. 667.

- encephalomyelitis and heredity. 430.
- infectious canine hepatitis. 239.
- influenza, cation exchange resin. 670.
- poliomyelitis, assay. 527.
- poliomyelitis, culture. 167.
- poliomyelitis, fluoroacetate effect. 83.
- poliomyelitis, intracerebral adaptation. 344.
- poliomyelitis, intracerebral inoculation. 454.
- poliomyelitis, mold filtrate effect. 104.
- poliomyelitis, tissue culture. 359.
- tobacco mosaic, iodine. 157.
- yellow fever, particle size. 613.

Vitamin, A metabolism, nephrectomized rats. 457.
- ascorbic acid, vanadium poisoning. 206.
- avidin preparation. 515.
- B, nutritional studies. 547.
- B_6, isoniazid relationship. 389.
  - deficiency. 441.
- B_{12} deficiency, high fat and protein diet. 451.
  - histological studies. 382.
  - protein catabolic action. 341.
  - transmethylation. 307.
- choline deficiency. 401.
  - metabolism, liver. 364.
  - toxicity. 642.
- deficiency, exophthalmos. 424.
- E and B_6 on leucocytes. 280.
- ethanol oxidation. 252.
- pantothenic acid deficiency. 521.
  - bacterial infection. 517.
- thiamine requirements of ciliate. 495.

X-radiation, antibody survival. 412.
- atabrine and leucopenia. 460.
- blood changes. 587.
- locus of emetic action. 472.
- testosterone, sex organs. 655.
- tissue mast cells. 306.
- tryptophan peroxide. 29.